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Molecular investigations of the nature, occurrence and behaviour of myo-inositol hexakisphosphate in soils using ion chromatography and high-resolution mass spectrometry

Molecular investigations of the nature, occurrence and behaviour of *myo*-inositol hexakisphosphate in soils using ion chromatography and high-resolution mass spectrometry

by

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Science

School of Chemistry

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Abstract

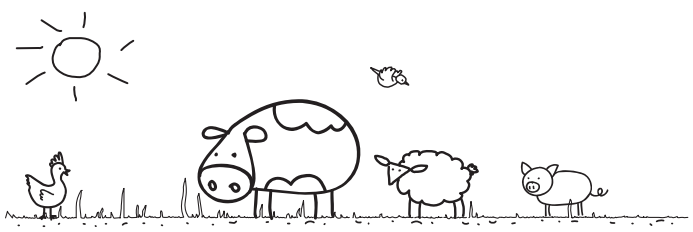
The global P biogeochemical cycle is important in the areas of agriculture and water quality. P is required as fertiliser for agricultural systems, and excess P in aquatic systems causes pollution by elevating nutrient levels, which can lead to eutrophication. The P cycle has been impacted by anthropogenic activity and rate of transport of P mined from rock sources to the ocean now exceeds that required for a safe operating space for humanity. The role of organic P compounds in the P biogeochemical cycle is poorly understood despite this fraction constituting up to 80 % of total P in some systems. Methods of characterisation of P in soil and aquatic systems are heavily reliant on bulk determinations of operationally defined "pools" of P. ^{31}P NMR characterisation of soil organic P has become widespread but is limited in its power to resolve molecular components.

This project aims to develop molecular methods of determining organic P in soil extracts, using the key soil organic P compound *myo*-inositol hexakisphosphate (IP6) as a model compound. The method developed identifies and quantifies IP6 isolated from complex matrices using ion chromatography (IC) and electrospray ionisation high-resolution mass spectrometry (ESI-HRMS). The unusual behaviour of this highly charge-dense compound in ESI-HRMS is characterised. The negative ion mass spectrum of IP6 displays a characteristic pattern of charge acquisition, fragmentation and adduct formation of value in confirming its presence in complex matrices.

Combining offline IC with ESI-HRMS, IP6 was identified in soil extracts, and its quantification by standard addition was compared with that from ^{31}P NMR experiments, with good correlation ($r = 0.9554$) achieved between the two methods. The IC/HRMS method was found to be faster, more sensitive and with lower sample requirements than ^{31}P NMR spectroscopy quantification. Investigating the extraction efficiency of NaOH-EDTA for IP6 (EEIP6) from a range of soils, it was found that individual soil characteristics correlated significantly with EEIP6. Extraction efficiency was found to be highest in soils with high total metal concentrations and low organic content. These relationships were not observed for the extraction efficiency of total P from soil.

The behaviour of IP6 in soil from poultry litter versus a solution of the compound was investigated in an incubation experiment. It appears that concentrations of soil IP6 from poultry litter, and to a lesser extent IP6 solution, increase initially over the first two weeks post application, before declining over the following weeks. The propensity of IP6 to bind to proteins in grains in the poultry diet appears to prevent extraction and soil microbial activity is required to decompose poultry litter and release the compound, before net degradation takes place. A pilot transport experiment using soil columns incubated with poultry litter for 1 day and 14 days confirmed this behaviour. IP6 from poultry litter appears to be equally mobilised by simulated rainfall regardless of incubation time.

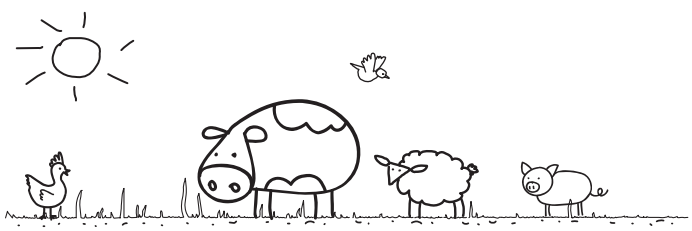
Together, these results indicated a dynamic behaviour of IP6 in soil, that is best investigated using the molecular methods developed here. Characterisation of organic P compounds using IC and ESI-HRMS can be developed further and applied to future investigations of the molecular nature of the P biogeochemical cycle, informing methods of management of human impact.



“Video” abstract

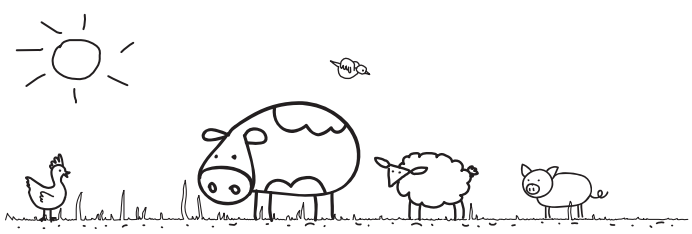
Dear reader, in the lower right-hand corner of the pages of this thesis you will find a video abstract in the form of a flipbook animation. The video begins on the last page and is played by flicking the pages from the back to the front. If you flip the entire book over the course of 10 seconds, you will have the frame rate about right.

Enjoy.



Dedication

Dedicated to my parents, Ken and Muriel McIntyre, who have been with me every step of the way with love and support.



Acknowledgements

What better time to write the acknowledgements than late in the evening, alone in the office, long after everyone else has left, exhausted and emotional, reflecting on the journey of the last four years (almost to the day). So many people who have smoothed the way here...

To start, of course none of this would have been possible without the excellent supervision and support of Prof Richard Evershed and Prof Penny Johnes. Thank you, Richard for providing the clarity in the fog of my thoughts and fostering the scientific rigour that I hope is evident in this work. To Penny for guiding me through the murky world of soil and water biogeochemistry. Then there's Dr Charlotte Lloyd. Where would I be without Charlotte? Always ready to talk through an idea and gently point out the, sometimes glaring, holes in my logic. It's been a pleasure to work with such interesting and interested scientists.

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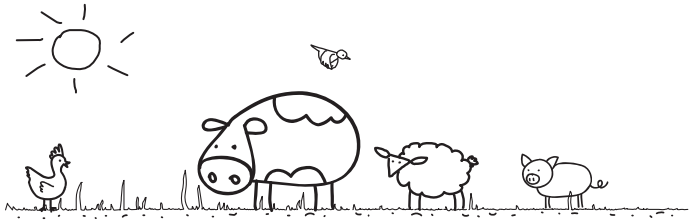
I am eternally grateful to my Dad, Ken, and sister, Fiona, for reading through this thesis and cross checking. But particular thanks go to Fiona (Dr Fiona) who has been my guide through the PhD student experience, on the other end of the phone explaining "Yes, that's normal!"

I have been supported financially by the National University of Ireland on a Travelling Studentship, and by NERC DOMAINE for research costs and I am grateful to both for the opportunity.

And so, to my front row cheerleaders. First and foremost, my family (parents, Fiona, Niamh, Ciarán, Conor and too many others to name) have been incredible. I couldn't have asked for a better family. Thanks guys! The legends of the OGU, Vittoria, Jerome and JP, thank you for support, beers and chats and making lab work such a joy. I have been lucky to have an amazingly supportive home team: Sinéad, Mary, Maria and Éimear, sending through motivational texts and always a joy to catch up with in Naas. Shout out also to the Post Brexit/Trump Fallout crew: Mick, Aoife, Sonja, Bianca and Chiara, on hand to let off steam! Thank you also to Karen and Hannah, Christian and Iain, and the early morning bootcamp crew for the shot of endorphins to set me up for the day. To Yas and Alasdair for all the fine food!

Thank you to everyone who has taken me for a beer, or a run, or a holiday, or fed me, or pointed out that I'm out by a factor of ten (again!). I couldn't have done it without you!



Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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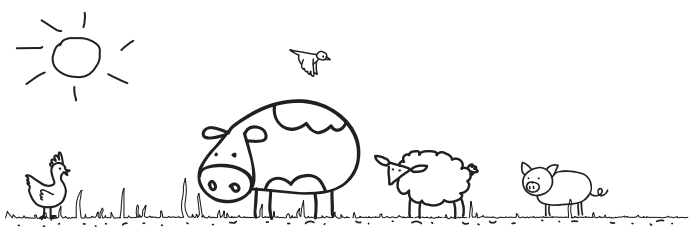

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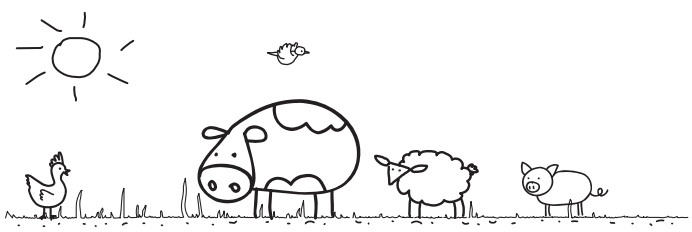

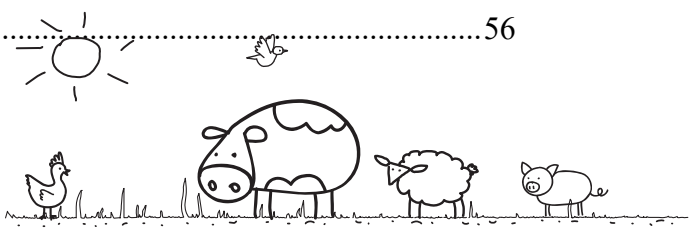

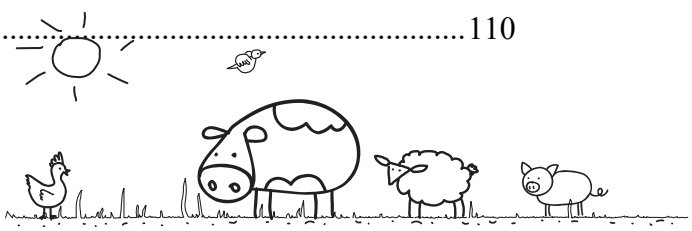

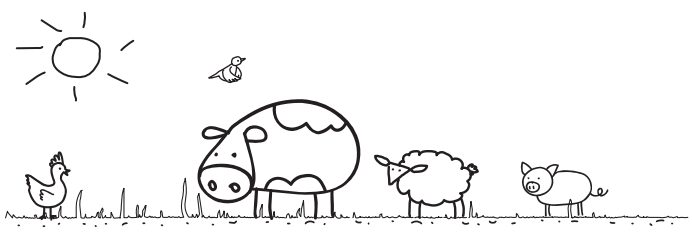

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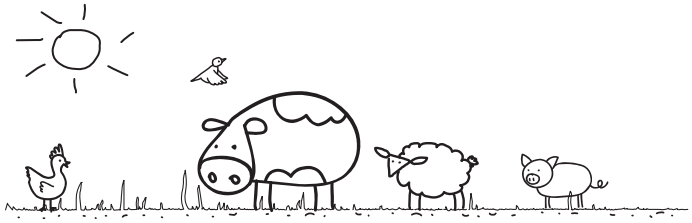

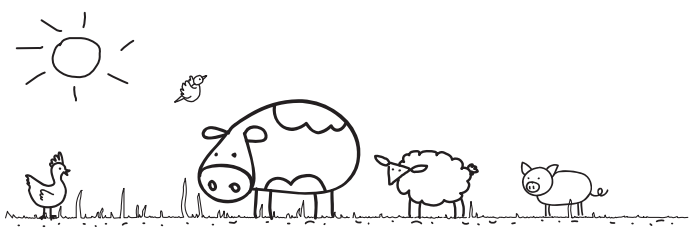

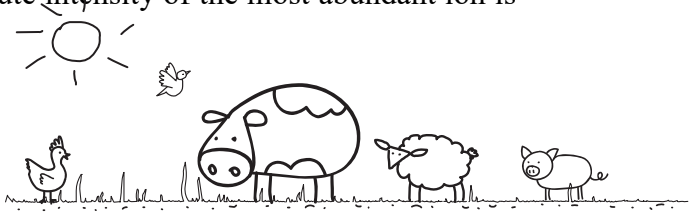

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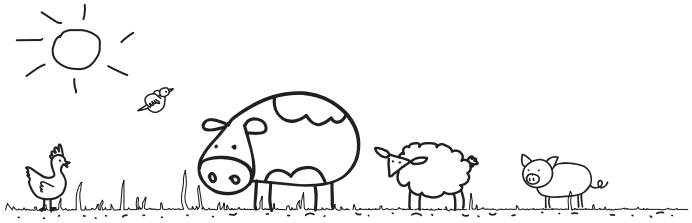
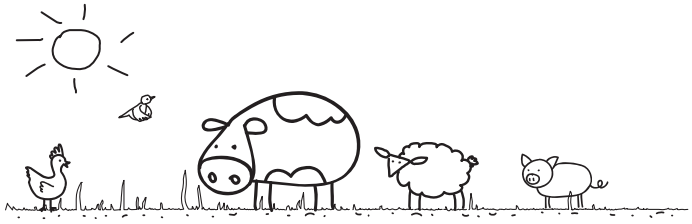


Abbreviations

A3MP	Adenosine 3’ monophosphate
A5DP	Adenosine 5’ diphosphate
AR	Arable
Arb	arbitrary units
ATP	Adenosine triphosphate
CTRL	Control
DDW	Double distilled water
DG6P	D-glucose-6-phosphate
DMC	Dry matter content
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
EAMP	Ethanolamine phosphate
EBPR	Enhanced biological phosphate removal
EDTA	Ethylene diamine tetraacetic acid
EEIP6	Extraction efficiency of NaOH-EDTA for IP6
EETP	Extraction efficiency of NaOH-EDTA for total P
ESI	Electrospray ionisation
FABMS	Fast atom bombardment mass spectrometry
FTICR	Fourier-transform ion cyclotron resonance
G5MP	Guanosine 5’ monophosphate
GC	Gas chromatography
GR	Grassland
HCD	Higher energy collisional dissociation
HPLC	High performance liquid chromatography
HPIC	High performance ion chromatography
HRMS	High-resolution mass spectrometry
IC	Ion chromatography
ICP-O(A)ES	Inductively coupled plasma optical (atomic) emission spectroscopy
IP5	*myo*-inositol pentakisphosphate
IP6	*myo*-inositol hexakisphosphate
IPx	Inositol phosphate (x substituents)
LC	Liquid chromatography



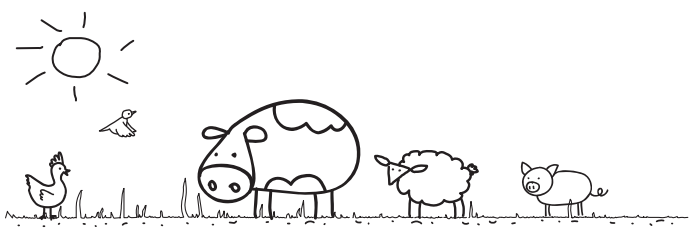
LOD	Limit of detection
LOI	Loss on ignition
LOQ	Limit of quantification
LSIMS	Liquid secondary ion mass spectrometry
M	Molar
MC	Moisture content
MDPA	Methylene diphosphonic acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFP	Methylumbelliferyl phosphate
NMR	Nuclear magnetic resonance
PAO	Phosphate accumulating organisms
PCRT	Phosphocreatine
PEP	Phosphoenolpyruvate
PF	Prior's farm
PL	Poultry litter
PS	Phytate solution
PTFE	Polytetrafluoroethylene
QH	Quidhampton
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SEC	Size exclusion chromatography
SIM	Selected ion monitoring
SL	Somerset Levels
SPE	Solid phase extraction
TP	Total P
WD	Woodland
WFD	Water Framework Directive
WHC	Water holding capacity

Chapter 1. Introduction

1.1 Organic phosphorus in the environment – global importance, biogeochemical cycle.

Phosphorus (P), along with carbon (C), oxygen (O) and nitrogen (N), is one of the essential elements for life, being a key constituent of DNA, the phospholipid bilayer of cell membranes, and the energy currency of the cell, adenosine triphosphate (ATP). Like C, O and N, it is subject to a global biogeochemical cycle. Figure 1.1 details the flow of P through the biosphere and geosphere, beginning from phosphate rock sources where it is mined for fertiliser and industrial uses. P is added to soils largely from application of phosphate fertiliser, both chemical and in organic forms, such as manure. P then cycles through microbes, plants and animals *via* uptake, transformation, excretion and decay. P is found concentrated in waste streams: manures, food waste, municipal and industrial waste effluents. Through discharge of waste streams, treated effluent, and run-off from agricultural soil, P is transported from the landscape to rivers and lakes. A proportion will be sequestered in sediments. Ultimately, P from the landscape is transmitted to the ocean where it contributes to ocean primary productivity and eventually sedimentation processes result in the formation of phosphate rock.

Although it is a cyclical process, unlike the C, O and N biogeochemical cycles, the P biogeochemical cycle has no atmospheric component. The timescale of recovery of P from ocean or sediment deposits is on the order of millennia, while most other processes in the cycle are on the order of seconds to decades. This creates a bottleneck in the global cycling of P, making it essentially a non-renewable resource in our lifetimes. Therefore, the increased rate of flow of P from rock to ocean water due to human interference in the P biogeochemical cycle is of great concern for the sustainability of P resources.



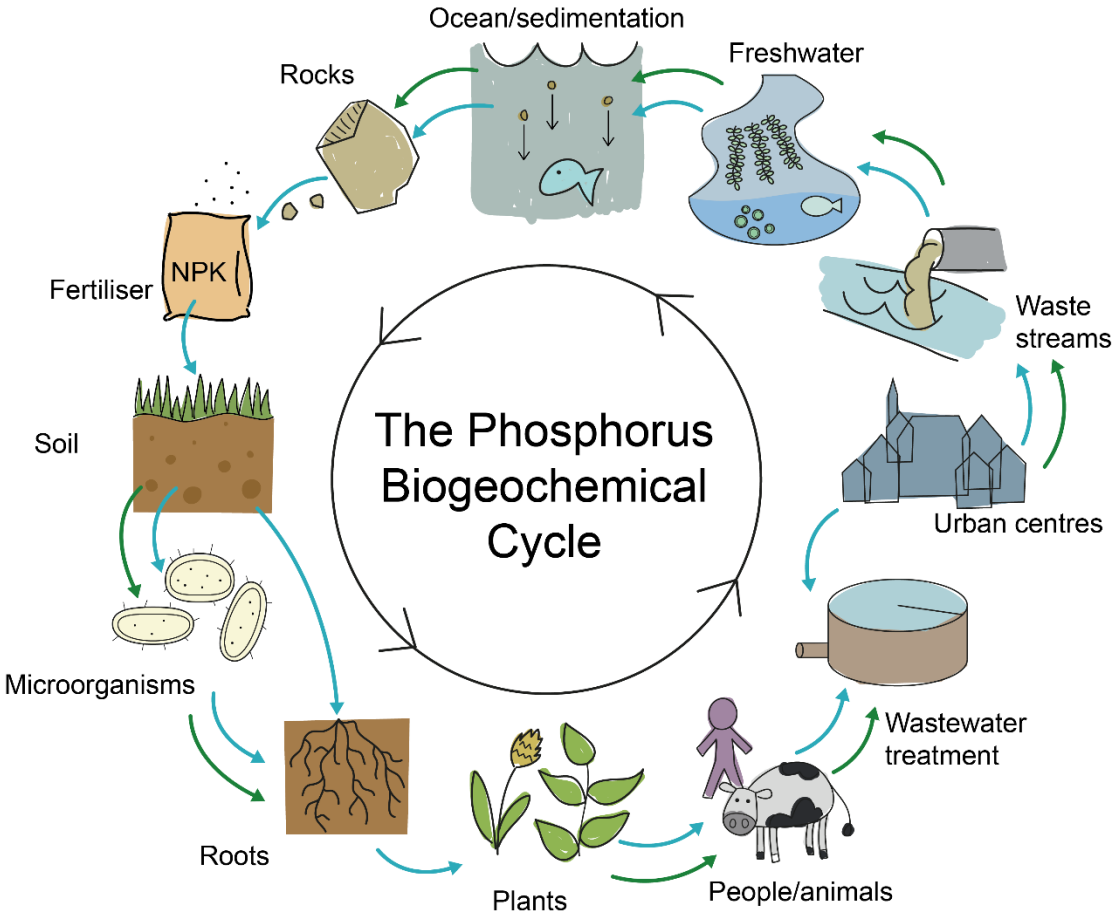


Figure 1.1. The global P biogeochemical cycle. Transport processes are indicated by blue arrows, and transformations processes by green arrows. A large proportion of P transport is from phosphate rock to agricultural land (see Figure 1.2). P is essential for every lifeform and is taken up, transformed and transported by living organisms before being concentrated in waste streams and transported or recycled.

Figure 1.2 depicts current flows of P resources globally. As can be seen, human interference in the P biogeochemical cycle by the extraction of rock phosphate for fertiliser accounts for 17.5 Mt yr^{-1}. Historically, P fertiliser was applied to farmland in the form of manure from local household or animal wastes, or from P-rich guano deposits (Ashley *et al.,* 2011). Upon discovery of the high P content of bird droppings, this valuable resource was shipped over large distances and thus began tipping of the balance of the global cycle. The Green Revolution in the 1950s and 1960s saw an explosion in the rate of extraction of phosphate rock for fertiliser (Figure 1.3) with a concomitant rise in food production per hectare and the world's population

(Cordell *et al*., 2009). Rock phosphate fertiliser is now overwhelmingly the largest source of P inputs to agricultural systems. Figure 1.3 shows how this has grown over the last century and indicates how reliant farming is on this cheap source of P.

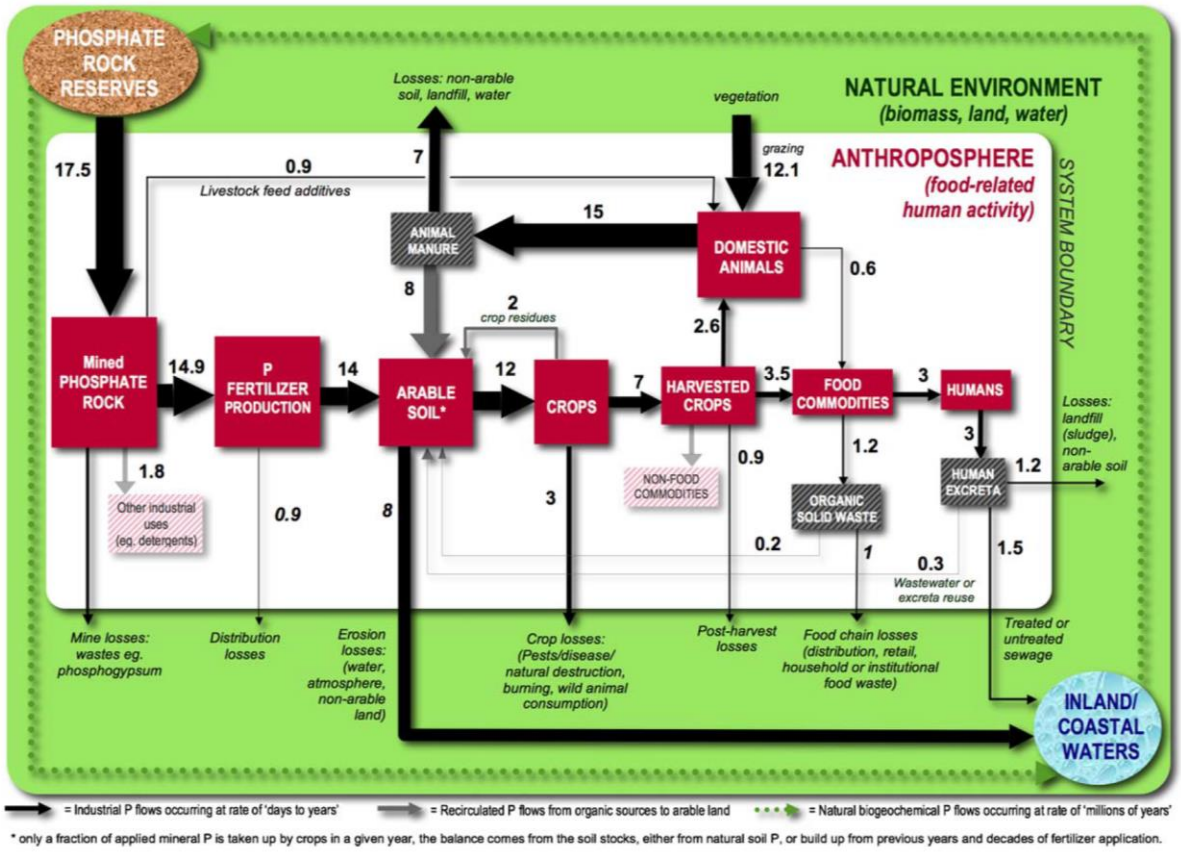


Figure 1.2. The quantities attributed to the global flow of P in Mt yr^{-1}. Losses are seen at each stage of food production and ultimately impact freshwater and marine systems. From Cordell *et al.* (2009).

Economically relevant deposits of rock phosphate are concentrated in just three major areas globally – Morocco, China, and the US (Cordell & White, 2014). Precisely how much phosphate rock is stored in each of these reserves is not known (Geissler *et al.*, 2018). Estimates that reserves could run out this century, with peak phosphate occurring as early as 2030 have been put forward (Cordell *et al.,* 2009) and disputed (Scholz and Wellmer, 2018). The uncertainty regarding the quantification of supplies of this important resource highlight the need for sustainable management of P fertilisers and an urgency to shift to the development of

methods of recycling P from waste for sustainable food production and prevention of water pollution (Rhodes, 2013, Cordell *et al.*, 2011).

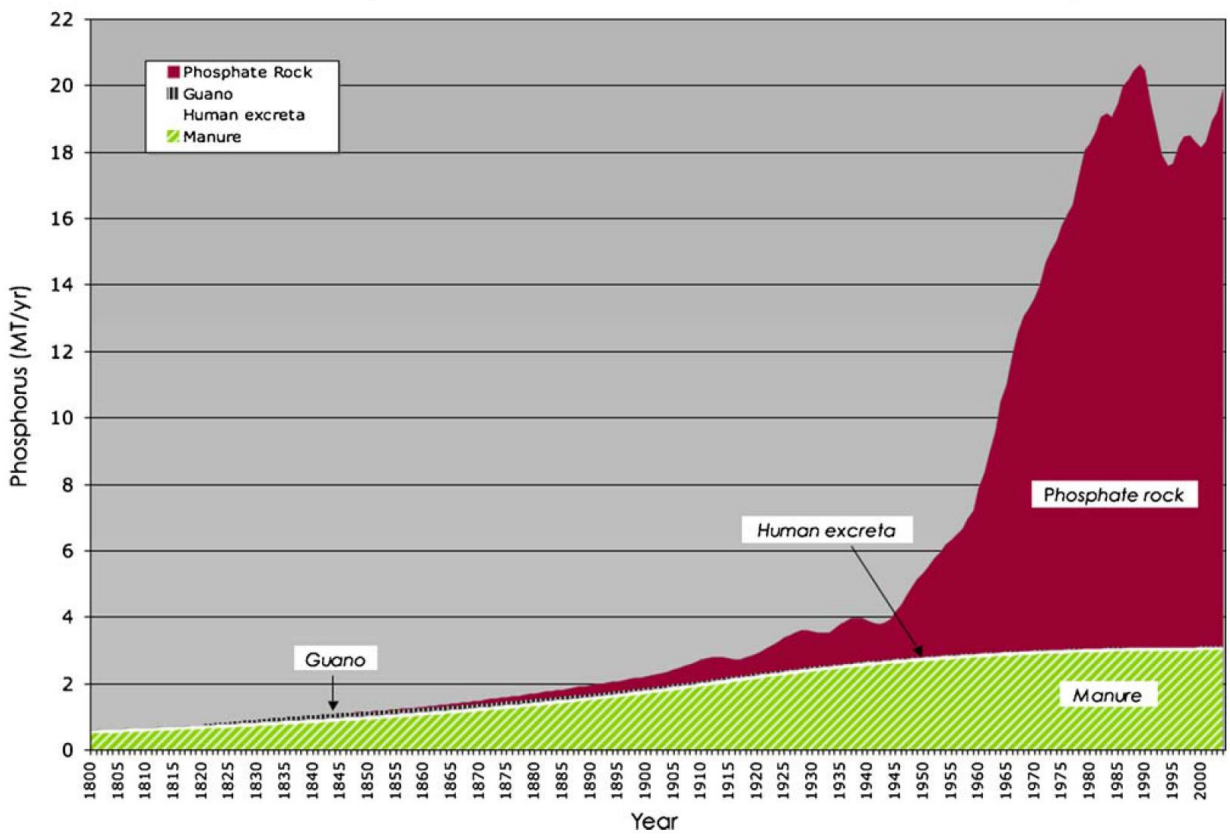


Figure 1.3. Historical global sources of P fertiliser (1800 – 2000). Sources indicated are: manure, human excreta, guano and phosphate rock. The acceleration in extraction of phosphate rock from 1940 – 1960 is striking and it now accounts for 90 % of global P fertiliser use. From Cordell *et al.* (2009).

Enhanced flow of P to waterways causes excess algal growth, leading to eutrophication and in extreme cases, such as the Great Lakes or the Gulf of Mexico, results in hypoxic zones, commonly known as dead zones (Jokinen *et al.* 2018; Diaz & Rosenberg, 2008; Sylvan *et al.*, 2006). These are largely due to transport of P from agricultural land and sewage effluent to coastal waters. The more frequent occurrence of excess P conditions and their increasing persistence is a cause for concern on both regional and global scales. Low oxygen conditions can enhance the release of P from ocean sediments, creating a self-sustaining cycle and potentially leading to the ocean anoxic events seen in past climates (Watson *et al.*, 2017).

Concern over the sustainability of current P resource use trends has led to the inclusion of P in the Planetary Boundary Framework devised by Rockstrom et al. (2009) and updated by Steffen et al. (2015). The aim of the framework is to calculate a threshold boundary for a range of Earth system parameters within which is a safe operating space for humanity. The boundary for global P flow was determined to be 11 Tg P yr^{-1}, which has already been exceeded at ~22 Tg P yr^{-1}. The boundary for P flow from fertilizers to erodible soils (6.2 Tg P yr^{-1}) has also been breached with ~14 Tg P yr^{-1} mined and applied to erodible soils. These breaches lead to uncertainty in the ability of the Earth's system to respond to this parameter. The Planetary Boundary Framework research highlights the need for urgent mitigation practices to reduce anthropogenic impact on the P biogeochemical cycle.

Measures to reduce the impact of P flow to freshwaters have been introduced in jurisdictions across the world. In the European Union the Nitrates Directive (91/676/EEC) regulates the application and the timing of application of fertilisers, including P fertilisers, in agriculture. The European Water Framework Directive (WFD, 2000/60/EC) was first introduced in 2000 and set thresholds for classification of the ecological status of surface waters from high to poor based on biological, chemical and physical factors. Limits are set for the molybdate reactive phosphorus (MRP) concentration in the water. MRP is determined spectroscopically by reaction between phosphate and molybdenum (discussed further below). The annual mean of MRP concentrations for each category in surface waters in the UK are shown in Table 1.1. Where alkalinity is high due to bedrock character, a higher threshold for MRP is allowed, as these waters are considered to have more capacity for absorbance of phosphate before detrimental effects are observed. These figures are the revised 2013 thresholds. Interestingly, the threshold concentration for "Poor" status implemented by Irish authorities is > 250 μg L^{-1}.

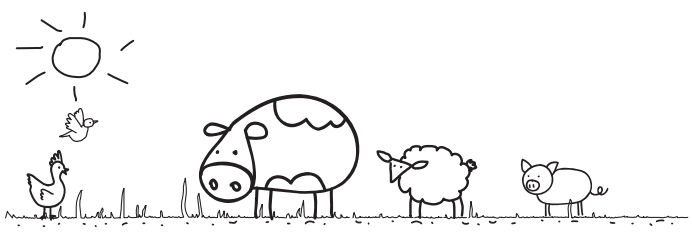


Table 1.1. Threshold annual mean concentrations for MRP for each status category of surface water in the UK in accordance with the EU WFD (DEFRA, 2014).

Type	Annual mean of reactive phosphorus (μg L^{-1})			
	High	Good	Moderate	Poor
Lowland, low alkalinity	19	50	150	500
Upland, low alkalinity	13	40	150	500
Lowland, high alkalinity	36	120	250	1000
Upland, high alkalinity	24	120	250	1000

Strategies have been developed in industry and farming to comply with the Nitrates Directive and WFD and in recognition of the advantages of recycling P resources. Farming practices include regular soil testing and reporting of P status of soils, limited use of fertiliser during the wet winter months, and introduction of buffer zones on farms between agricultural activity and waterbodies. Monitoring programs have been established on rivers and lakes across Europe. Initiatives such as the Demonstration Test Catchment (DTC) program determine the effectiveness of mitigation practices on the contribution of point and diffuse sources to the nutrient status of aquatic systems.

To reduce point source P in sewage outflow, P-stripping technologies have been introduced, including methods of P recovery for reuse as fertiliser. The most common and least expensive form of P-stripping in wastewater treatment is chemical treatment with ferrous iron. Iron (Fe III) chloride solution is added to effluent prior to discharge, forming a precipitate with free PO_4^{3-} in solution (Yeoman *et al.,* 1988). This precipitate is collected and sent to landfill. Recycling of the P by releasing it from the iron precipitate is prohibitively expensive. The iron content of the precipitate is too high for application to land and so the P resource is lost. Alternatively, P is biologically removed from wastewater in anaerobic digestion by the action of phosphate accumulating organisms (PAOs) in a process called enhanced biological phosphorus removal (EBPR) (Bashan & Bashan, 2004). Briefly, PAOs absorb P-containing compounds in waste streams and convert it to polyphosphate stored in their cells. Changing the

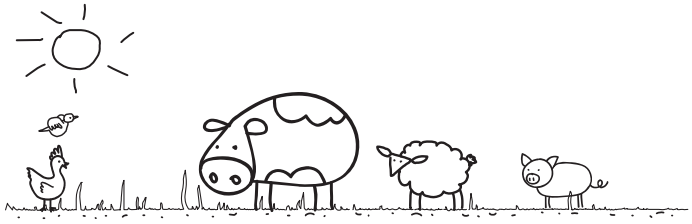
conditions of the reactor causes the PAOs to emit the polyphosphate and it is then precipitated with aluminium and magnesium forming a salt called struvite ($NH_4MgPO_4 \cdot 6H_2O$). Unlike $Fe_3(PO_4)_2$ precipitate formed by chemical P-stripping, struvite makes a very suitable fertiliser and thereby P is recycled.

These measures aimed at the reduction of anthropogenic flow of P to water bodies, such as the WFD thresholds and P-stripping strategies, primarily target the inorganic portion of total P. However, organic P compounds can constitute up to 80 % of total P in some soil systems (Harrison, 1987). Understanding the organic P compounds of the P biogeochemical cycle and the nature of their transformation and transportation processes is essential to ensuring that mitigation practices are effective, and that human interference is brought under control. However, there is currently limited knowledge of the nature of organic P compounds and their contribution to agricultural productivity or water pollution.

1.2 Structure of organic P compounds

Organic P covers a wide range of compounds which vary in structure, polarity, chemical reactivity and abundance. The phosphate (PO_4^{3-}) moiety dominates the chemical structure of natural organic P compounds. Yet, the substituent esters of the phosphate group can vary greatly in form and structure. For example, phospholipids contain long alkyl chains imparting apolarity to the majority of the compound. Inositol phosphates (discussed in more detail later) are compounds with high electron density and occur as readily charged species in most matrices.

The three most commonly occurring P compound classes are shown in Figure 1.4, including phosphate monoesters, phosphate diesters, polyphosphates and phosphonates. Phosphate monoesters have a single P-O-C ester bond, phosphate diesters have two ester bonds, organic polyphosphates have multiple phosphate moieties bound in series to the organic structure, and



phosphonates are characterized by a direct P-C bond. Examples of these compounds found in nature are D-glucose-6-phosphate, a phosphate monoester which is the phosphorylated form of glucose transported across cell membranes for uptake of the carbon source. The post translational modification of proteins by phosphorylation, results in peptide phosphate monoesters. The diesterification of the ribose phosphate of nucleotides forms the backbone of the helical structure of DNA. Phospholipids also take a diester (and sometimes monoester) form. The energy storage compound adenosine triphosphate (ATP) is formed by the sequential phosphorylation of adenosine to adenosine monophosphate, diphosphate and triphosphate. Phosphonates are in low abundance in terrestrial natural systems but are thought to be important in marine P cycling, providing a source of P for microbial primary producers (Dyhrman *et al*., 2006).

There are less common organic P compound structures, such as phosphazenes like phosphocreatine, which have a P−N bond. Anthropogenic organic P compounds such as pesticides and pharmaceuticals often have these more atypical structures (Figure 1.5). P=S or P-S (phosphorothionates), P=N, P−N (phosphamides or phosphazenes) bonds may be found in these compounds.

The electronegativity of P is equivalent to that of hydrogen (H) at 2.1. Therefore, the polarity of the P-O and P=O bonds result in the PO_4^{3-} moiety being a highly electronegative, often charged, environment on P compounds. It is this strong polarity that gives P compounds their functional properties, for example in promoting biological reactions *via* the phosphorylation of substrates by ATP; and forming the hydrophilic polar heads of membrane lipids.

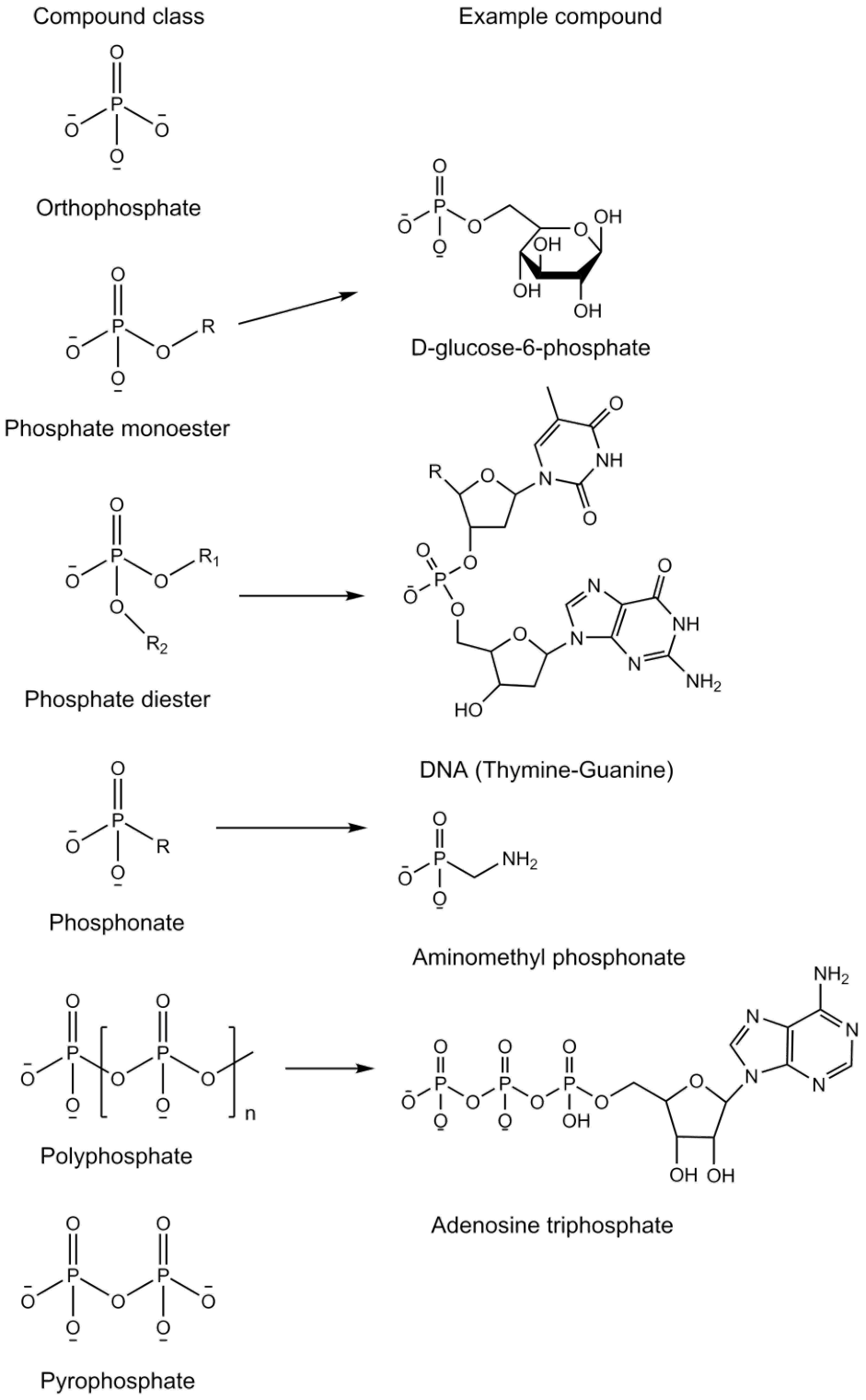


Figure 1.4: Structures of the most common P compound classes. LHS shows the basic structure of each compound class. Orthophosphate and pyrophosphate are inorganic P compounds. P monoesters have a single P-O-C bond, P diesters have two P-O-C bonds, and phosphonates have a direct P-C bond. Polyphosphates can be either organic or inorganic. RHS shows examples of compounds for each class.

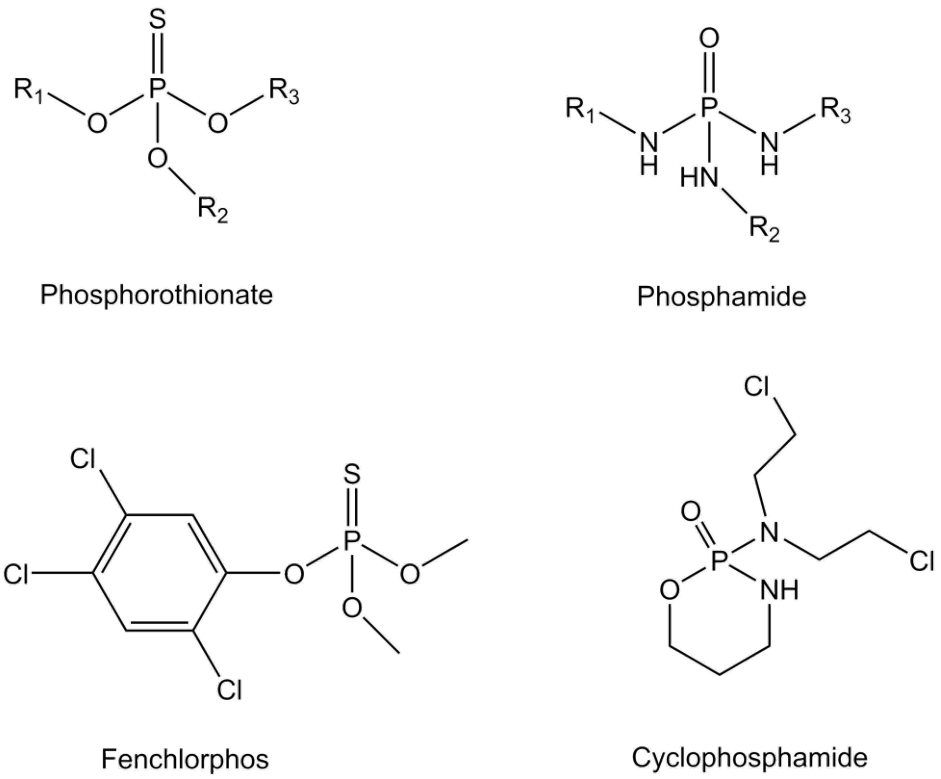


Figure 1.5. Examples of structures of anthropogenic forms of organic P. Fenchlorphos is an insecticide and cyclophosphamide is an anti-cancer drug. Many more varieties exist; however, they occur in very low abundance in the environment.

1.3 Organic P in the environment

Concentrations of the compound classes in a range of matrices are given in Table 1.2. Aside from point sources such as wastes, soils are the major terrestrial source and sink of organic P compounds. It is the primary recipient of phosphate rock fertiliser and the link between agricultural inputs and aquatic systems. Therefore, the binding of P to soil, and the transformation and transportation processes it undergoes in soils have major implications for the global P biogeochemical cycle. Understanding the rates and of mechanisms of organic P processing in soil is vital to learning how to manage human influence on the P biogeochemical

cycle, to enable sustainable management of P resources, and to minimise pollution to waterbodies.

1.4 Abundance, cycling and binding of P to soil

Inorganic phosphate is frequently the most abundant form of P in soil, forming salts with mineral cations such as Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, although the organic P fraction can often dominate in weathered and forest soils (Hedley *et al.,* 1982; Turner & Engelbrecht, 2011). The range of proportions of each of orthophosphate, phosphate monoester, phosphate diester, pyrophosphate, polyphosphate, *myo*-inositol hexakisphosphate (IP6) and total P in soils, sediments and manures according to literature studies are shown in Table 1.2. Menezes-Blackburn *et al.* (2018) performed Bootstrap statistical analysis on data from ^{31}P NMR spectroscopy studies of agricultural soil extracts in 41 publications and compiled the summary of typical total soil P stocks in kg P ha^{-1} for the first 15 cm of soil shown in Table 1.3. Organic P frequently forms a substantial proportion of soil P. Of this, IP6 is found to make an important contribution, up to 58 % of organic P (Turner, 2007). The individual organic P compounds that are commonly determined in NMR or enzymatic hydrolysis assays are generally biological P compounds derived from microorganisms as detailed in Table 1.4. However, microbial P has been calculated to represent just 8 % of soil P globally (Xu *et al.,* 2013).

Little is known about the forms or quantities of organic P compounds besides the microbially-derived compounds listed below. Using ultrafiltration, McLaren *et al*. (2015a) separated ten soil extracts by size into fractions of > 10 kDa and < 10 kDa. ^{31}P NMR analysis revealed a large proportion (61 – 73 %) of soil organic P in the form of phosphate monoesters within supra- / macro-molecular structures termed “humic P”. Concentrations of these unknown compounds were between 22 and 324 mg kg^{-1}. The challenges in analysing this suite of compounds are outlined below.

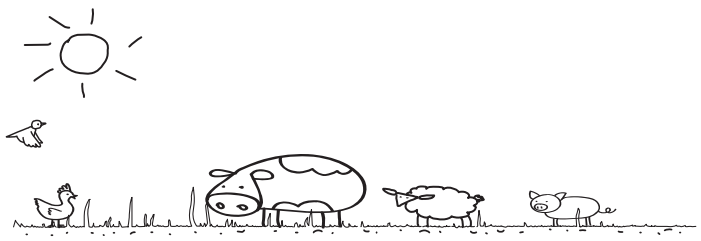


Table 1.2. Range of concentrations of compound classes in soils, lake sediments and manures. Orthophosphate (Ortho), phosphate monoesters (P-mono), phosphate diesters (P-di), pyrophosphate (pyro-P) and polyphosphate (poly-P) are given in percentages and IP6 and TP in mg P g^{-1} concentrations.

Matrix	P-compound class						
	Ortho	P-mono	P-di	pyro-P	poly-P	IP6	TP
	%					mg P g^{-1}	mg P g^{-1}
Soils[1,2,3]	26 - 86	11 - 71	1 - 9	ND - 9	ND - 3	0.001 - 0.220	0.116 – 2.746
Lake Sediments[4,5]	38 - 42	35 - 37	15 - 20	0 - 12	0 - 10	0.024 - 0.149	0.25 - 0.90
Broiler litter[6,7]	34 - 49	47 - 66	< 4.5	trace	ND	4.1 - 8.2	15.3 - 15.5
Cattle manure[6,8]	65 - 67	15 - 23	4 - 11	3 - 7	6 - 8	0.2 - 10.8	4.1 - 4.5
Swine manure[6,9]	87 - 90	8 - 10	1 - 2	0.3 - 0.4	< 0.3	1.9 - 14.3	12.7 - 13.4

References: [1]Murphy *et al.* (2009), [2]Hill & Cade-Menun (2009), [3]McDowell *et al.* (2005), [4]Reitzel *et al.* (2006), [5]de Groot & Golterman (1993), [6]Turner (2004), [7]Leytem *et al.* (2008), [8]Ray *et al.* (2012), [9]Leytem & Thacker (2010).

Table 1.3. Soil phosphorus stocks analysis of global literature on 258 agricultural soils in 41 publications using ^{31}P NMR spectroscopy. Soil bulk density was used to transform data from mg kg^{-1} into kg ha^{-1}. From Menezes-Blackburn *et al*. (2018).

	Total P	Inorganic Orthophosphate		Monoester		Diester		Other		
	kg ha^{-1}	kg ha^{-1}	(%)	kg ha^{-1}	(%)	kg ha^{-1}	(%)	kg ha^{-1}	(%)	n
All samples	1762 ± 132	1006 ± 115	(57 ± 7)	587 ± 32	(33 ± 2)	64 ± 7	(4 ± 0)	96 ± 13	(5 ± 1)	258
Arable soils	1666 ± 133	964 ± 72	(58 ± 4)	519 ± 62	(31 ± 4)	64 ± 15	(4 ± 1)	123 ± 28	(7 ± 2)	115
Pastures	1830 ± 220	1037 ± 190	(57 ± 10)	644 ± 28	(35 ± 2)	64 ± 6	(3 ± 0)	74 ± 6	(4 ± 0)	143
Europe	1699 ± 94	927 ± 82	(55 ± 5)	646 ± 28	(38 ± 2)	55 ± 7	(3 ± 0)	68 ± 7	(4 ± 0)	143
North America	2170 ± 327	965 ± 94	(44 ± 4)	842 ± 177	(39 ± 8)	129 ± 42	(6 ± 2)	250 ± 81	(12 ± 4)	35
Oceania	1947 ± 412	1350 ± 363	(69 ± 19)	472 ± 36	(24 ± 2)	44 ± 8	(2 ± 0)	92 ± 14	(5 ± 1)	75

Table 1.4. Chemical group, NMR region, examples, location and function of main forms (quantitatively) of P in microorganisms. From Bünemann et *al.* (2011).

Chemical group	NMR region	Examples	Description
Nucleic acids	Diester	DNA, RNA	Biopolymers present in all living cells; storage of genetic information. About 9% P
Phospholipids	Diester	Phosphatidylglycerol, phosphatidylethanolamine	Major components of cell membranes; ensure semipermeability. Usually one phospholipid molecule consists of glycerol, two saturated or unsaturated fatty acids, and one P atom
Teichoic acids	Diester	Glycerol teichoic acid	P-containing polysaccharides present in cell walls of Gram-positive bacteria
Metabolites	Mostly monoester	AMP, glucose-6-phosphate, phosphoenol pyruvate, ATP	Intermediates and products of all metabolic processes
Phosphonates	Phosphonate	2-Aminoethyl-phosphonic acid	Compounds with direct bonds between carbon and P atoms. Biological function not well known
Organic polyphosphates	Pyro- and polyphosphate	ATP	Produced by ATP synthase. Transfers chemical energy when converted back to AMP or ADP and orthophosphate
Inorganic pyrophosphate	Pyrophosphate	Pyrophosphate	P storage compound consisting of two condensed phosphate groups
Inorganic polyphosphates	Polyphosphate	Polyphosphate	P storage compounds consisting of three or more condensed phosphate groups
Orthophosphates	Orthophosphate	HPO_4^{2-}; $H_2PO_4^-$	Inorganic anions of phosphoric acid. Used in metabolic processes and for synthesis of complex molecules

DNA deoxyribonucleic acid, *RNA* ribonucleic acid, *AMP* adenosine monophosphate, *ADP* adenosine diphosphate, *ATP* adenosine triphosphate

1.4.1 Binding

A range of characteristics, from the texture of the soil and the physical and chemical properties, influence the retention, transportation and processing of P compounds in soils. Clay, minerals, pH and organic matter all play a role in sorption of P (McKercher & Anderson, 1989; Celi *et al.*, 1999, 2000). Fine soils that retain moisture enhance P binding to soil and minimise leaching (Weihrauch & Opp, 2018). In basic soils, P is bound in Ca-P-minerals. In acidic soils Al-P-minerals are the primary form at mildly acidic conditions (~ pH 5), whereas Fe-P-minerals dominate below pH 4.5 (Anderson *et al*., 1974). Organic matter in soils appears to retain organic P compounds (Borie & Zunino, 1983). IP6 and the lower IPxs (described in more detail below) are frequently found to be the most strongly sorbed to soils, followed by inorganic phosphate, then lower order organic P monoesters.

Binding of organic P to soil is *via* the oxygens of the PO_4^{3-} to the metal cations of the mineral phase. Where a compound, such as IP6 has multiple PO_4^{3-} - groups, the binding is strengthened by the interaction of up to three PO_4^{3-} groups (Ognalaga *et al.,* 1994), leading to the stronger retention of compounds with multiple phosphate groups than that seen for phosphate (Anderson *et al*., 1974). The binding of diesters to the mineral phase is weakened by the second constituent on the phosphate group and these are more easily subject to degradation by enzymatic hydrolysis. Despite a higher input of P diesters to soils than monoesters from decaying organisms, their ready degradation means that monoesters are the dominant organic P compound class found. Metals, such as Fe and Al, may form bridges between P compounds and high molecular weight organic compounds (Gerke, 2015). The strong binding of P to soils, and hence lack of bioavailability is considered the reason for its accumulation, and particularly the preferential accumulation of the more strongly retained P compounds such as IP6 (Stewart & Tiessen, 1987). Between just 1.5 and 11 % of total soil P is considered readily available to

plants (Menezes-Blackburn *et al.,* 2018) and understanding the nature of the remaining P is a major obstacle to the immobilisation of this resource to reduce agricultural reliance on chemical fertiliser.

1.4.2 Biotic strategies for acquisition of soil P

P is taken up from the environment by organisms in the form of phosphate, and therefore organic P compounds must be mineralised to PO_4^{3-} before utilisation. Microorganisms and plants use extracellular enzymes, termed phosphatases, to hydrolyse complex organic P compounds, releasing the PO_4^{3-} into solution. Before mineralisation and uptake, PO_4^{3-} and organic P must be mobilised from the soil matrix. Plant roots and ectomycorrhizal fungi exude low molecular weight acidic compounds such as oxalic and citric acid to solubilise soil-bound P (Gerke, 1992). These dicarboxylates compete with the PO_4^{3-} moiety bound to soil particles, thereby releasing the P to solution. Despite having evolved these strategies for acquisition of soil P, mineralisation of organic P and diffusion of phosphate is a slow process (Barber *et al.,* 1963), and sizeable portions of the soil P pool are not considered readily available to plants and microbes (McDowell *et al*., 2007). This leads to application of fertiliser containing mobile PO_4^{3-} forms, which quickly bind to the soil matrix and accumulate. Finding ways to access this "legacy" P is one strategy for the reduction of reliance on PO_4^{3-} rock for fertiliser.

1.5 Inositol phosphates

The inositol phosphates (IPx) are found widely in natural environments and are present in high abundance in soils, sediments and manures. *Myo*-inositol is a cyclical sugar compound comprised of six carbon stereocentres. It is found widespread in eukaryotes and is an important signalling compound in multiple biochemical pathways (Loewus & Murthy, 2000). *Myo*-inositol hexakisphosphate, more commonly known as phytic acid (IP6), is reportedly the most abundant organic P compound in soils, sediments and manures and accounts for the largest

fraction of the inositol phosphates in these matrices (De Groot & Golterman, 1993). IP6 (Figure 1.6) is a highly unusual compound. Its organic property is conferred by the carbon-based inositol ring, however, the six phosphate substituents on these carbons impart highly electronegative properties to the compound. It is deprotonated above pH 1.1 and can carry up to 12 negative charges, being fully deprotonated at pH > 12 (Costello *et al.,* 1976). The compound adopts a 5 axial/1 equatorial conformation (Figure 1.6) at pH 1.0 – 9.0 (Barrientos & Murthy, 1996) and flips to the 1 axial/5 equatorial conformation at pH > 9.5, although this may be influenced by counter ions bound to the phosphate groups (Martin & Evans, 1986). Barrientos and Murthy hypothesise that the 5 axial/1 equatorial conformation is stabilised by hydrogen bonding between the phosphate groups or binding *via* sodium counter ions.

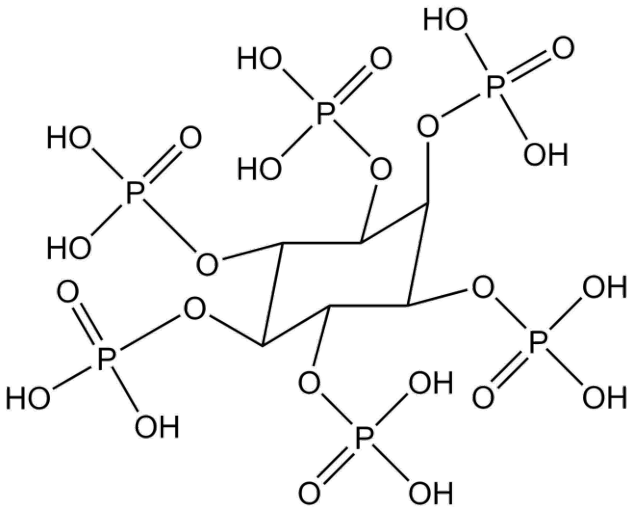


Figure 1.6. IP6 in the chair conformation. The structure is crowded with six phosphate groups around a 6-member carbon ring. There are 12 ionisable protons on the compounds giving it the potential to be a highly negatively charged species.

Inositol phosphates may have between one and six phosphate groups, these are called the lower IPxs and are denoted here by IP1, IP2, IP3, IP4 and IP5. The lower IPxs have all been detected in soils (Martin & Wicken 1966; Halstead & Anderson 1970), however, IP6 typically represents >80 % of the IPxs according to Anderson & Malcolm (1974). Stereoisomers of IP6,

i.e. D/L-*chiro*-inositol hexakisphosphate, *neo*-inositol hexakisphosphate and *scyllo*-inositol hexakisphosphate (Figure 1.7) are also found in soils, albeit in much lower concentrations. The order given by Turner *et al*. (2002) is *myo* > *scyllo* > D-*chiro* > *neo*, with *myo* representing 90 % of total IP6 (McKercher & Anderson, 1968). The sources of the stereoisomers are not known, but they are thought to be of microbial origin (Cosgrove, 1969, L'Annunziata, 1975).

IP6 is the plant P-storage compound found in high concentrations in seeds and to a lesser extent in roots. Within the seed tissue it accumulates in globoid crystals in the cells' protein bodies (Lott *et al.,* 1995) and can constitute up to 70 % of P in seeds. Animals fed on grains have a diet high in IP6, and their ability to digest the compound influences the quantities found in their faeces. Pigs and poultry, for example, lack phytase and therefore IP6 passes through their system intact and appears in high concentration in the pig manure (Cheryan, 1980; Humer *et al.,* 2015). The lack of availability of P from IP6 in the grain consumed by the animal means a lower P intake in their diet and farmers will supplement their feed with either phytase to release P, or phosphate salts (Simons *et al.,* 1990). Animal manure fertiliser, particularly that from grain-fed animals, is an important source of IP6 to soils.

IP6 may also be produced by soil microbes, although there is currently no direct evidence for this and plant material is considered the major input of IP6 to soils (Turner *et al*., 2002). IP6 in soil is broken down extracellularly by enzymes called phytases which are produced by plants and fungi and range in their specificity for inositol phosphates. As a carrier of six phosphate groups, the hydrolysis of IP6 releases phosphate to its surroundings, which may then be assimilated by plants or microbes or transported in solution promoting primary productivity in waterbodies. However, the strong sorption of IP6 found in numerous studies is thought to render the compound unavailable for degradation by organisms and to accumulate in soils (McKercher & Anderson, 1989; Celi *et al.,* 1999). This would explain the relatively high

abundance of the compound found in soil extracts. The binding of IP6 to soil particles suggests that the compound, though highly soluble in water, is transported in the particulate fraction, rather than *via* soil solution (Turner *et al*., 2002). Accumulation of IP6 in river and lake sediments is due to the settling of colloidal material out of the water column and subsequent build up.

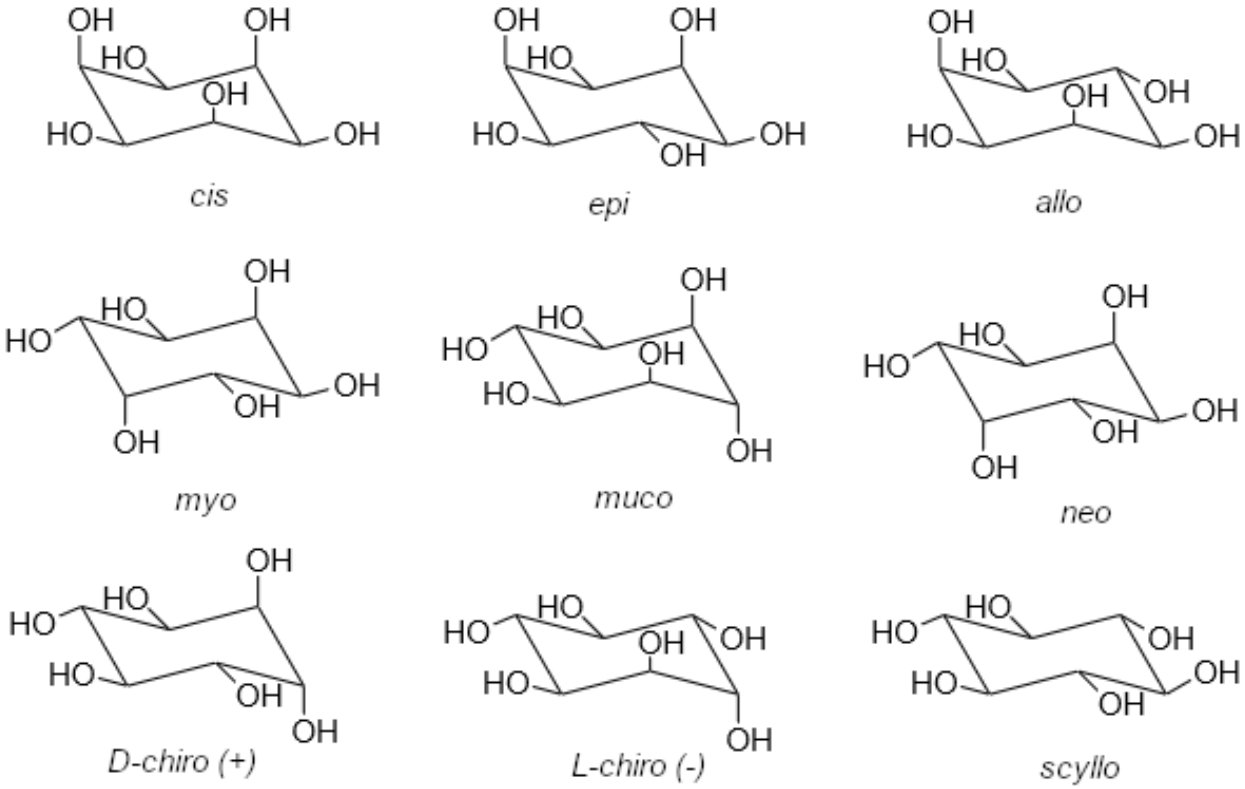


Figure 1.7. The range of stereoisomers of inositol. Phosphorylated forms e.g. *scyllo* hexakisphosphate are also found in environmental matrices, although in low abundance. Adapted from Turner *et al.* (2002).

Much of the research into organic P in soils and sediments has focussed on the dynamics of IP6 and the IPxs (Doolette *et al.,* 2010; Sjöberg *et al.,* 2016). As the most abundant organic P compound, it is readily detected in soil extracts *via* NMR (Turner *et al.,* 2003b). However, the unusual chemical structure of the compound and its highly electronegative properties make

isolation and purification of the compound extremely difficult and pure standards are not available. Detection and identification of IP6 is challenging and is discussed further below.

1.6 Analysis of P in environmental matrices

1.6.1 Analysis of P in water and soils

The Molybdenum Blue Test has been the standard method of P determination since its development in the 1960s. It involves the reaction of PO_4^{3-} with ammonium molybdate to form a Keggin structure according to reactions 1 and 2 (Nagul *et al.,* 2015):

$$PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O \quad (1)$$

$$H_3PMo(VI)_{12}O_{40} + \text{Reductant} \rightarrow [H_4PMo(VI)_8Mo(V)_4O_{40}]^{3-} \quad (2)$$

The concentration of P in solution is then determined by the Beer-Lambert law, by spectrophotometrically measuring absorbance at 880 nm. More complex organic P compounds are not reactive to molybdenum in this assay and are therefore digested with perchloric acid in order to release the PO_4^{3-} moiety for determination. The difference between PO_4^{3-} concentrations before and after digestion is termed “unreactive phosphorus”. Currently, the characterisation of aquatic P is by fractionation into components by operational definition. Total P (TP) is divided into soluble and particulate P by filtering to 0.45 μm. These are further defined by their reactivity (or not) to the Molybdenum Blue Test. The division of Total P into its constituent fractions is illustrated in Figure 1.8.

The reactive P and unreactive P are often referred to as the operationally defined categories of inorganic and organic P, respectively. However, there are some known disadvantages to defining P in this manner using the Molybdenum Blue Test (McKelvie *et al.*, 1995). Some

compounds, such as phosphonates and phospholipids, are known to be recalcitrant to the perchloric acid digestion and therefore the concentration of Total and "organic" P can be underestimated (Kerouel & Aminot, 1996, Monaghan and Ruttenberg 1999). On the other hand, some organic P compounds are labile under the conditions of the test and are thus counted in the "inorganic" fraction (McKelvie *et al.*, 1995).

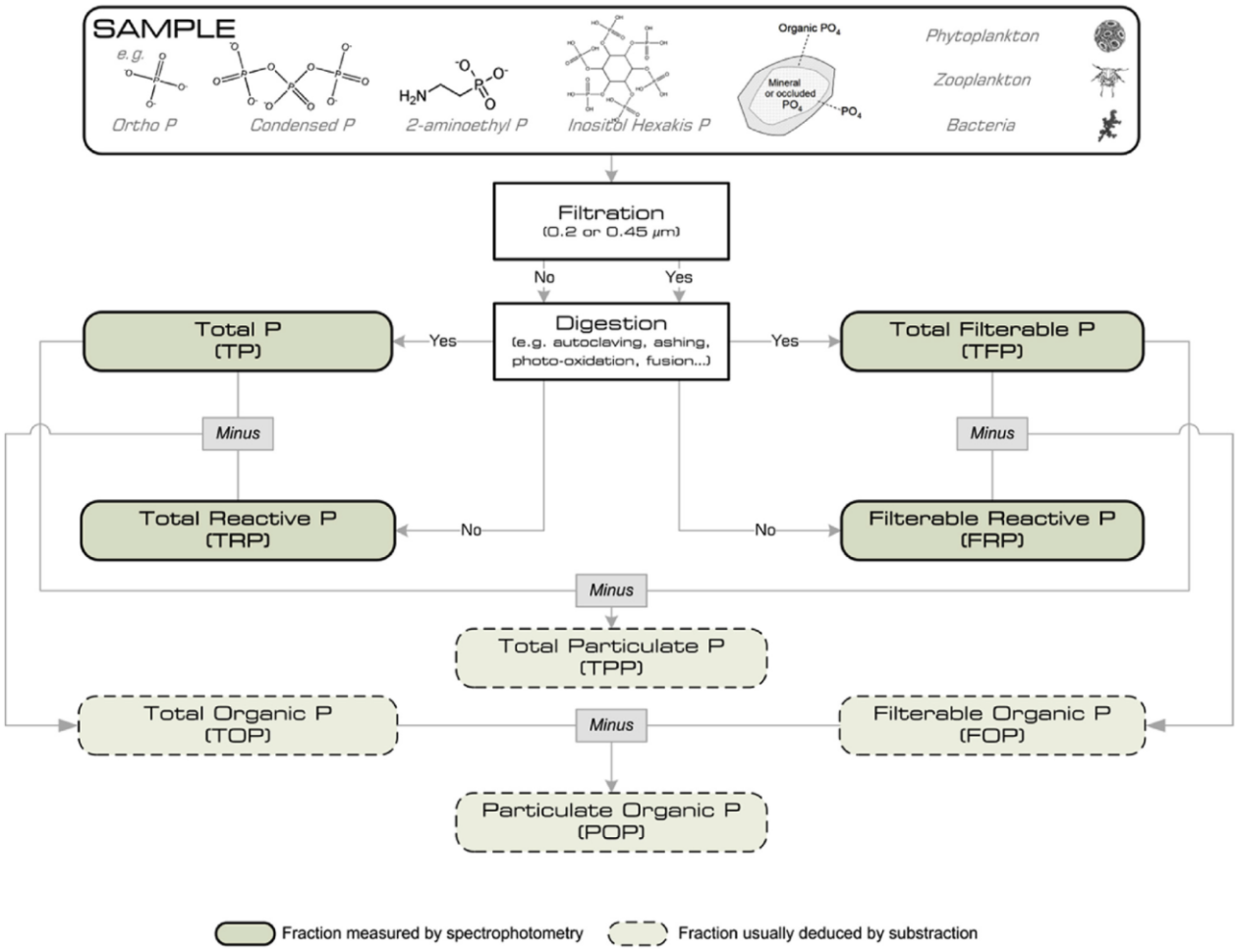


Figure 1.8. A schematic of the methods of characterisation of pools of aquatic P by operational definition. From Worsfold *et al.* (2016).

1.6.2 Soil fractionation

The Molybdenum Blue Test has been used in soil testing to determine PO_4^{3-} concentrations in soil extract solutions. Using a suite of extraction solutions of increasing strength, the

availability/recalcitrance of P pools is inferred (Hedley *et al.,* 1982). For example, a modified fractionation protocol is given in Figure 1.9. Variations of this fractionation scheme have been developed over the years, but the principle remains the same; extract and determine PO_4^{3-} concentration using the molybdenum blue test at each step.

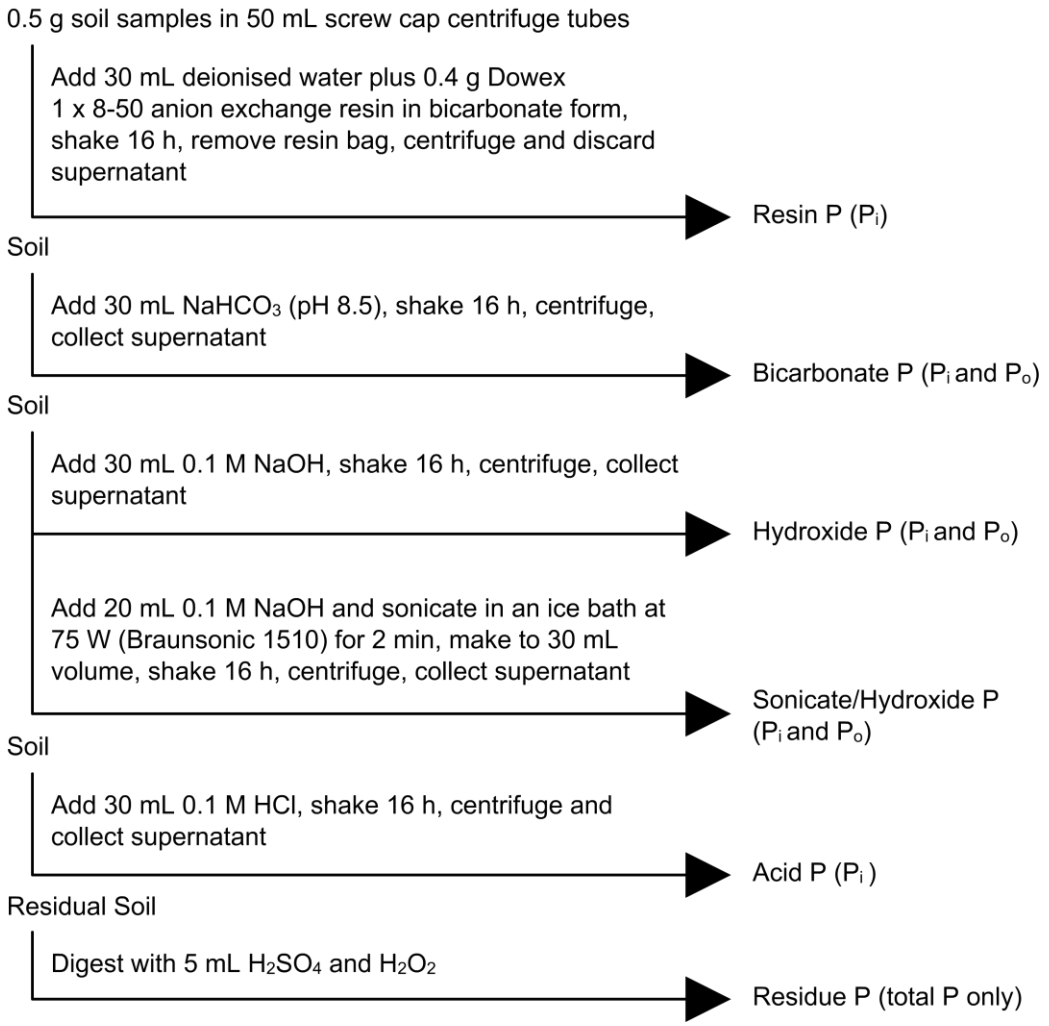


Figure 1.9. The method of sequential fractionation of soils based on the Hedley fractionation. Adapted from Tiessen *et al.* (1984).

Soil fractionation can be a useful tool in the characterisation of soils, or changes in soils after treatments, however, it is not possible to determine the molecular character of the P compounds in each fraction. The more easily extracted P fractions, e.g. those extracted using $NaHCO_3$, are considered to be more available to plants (Olsen *et al*., 1954). What physicochemical properties

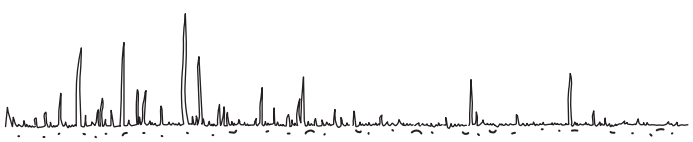
makes these compounds more bioavailable, or indeed if they truly are more bioavailable than the P compounds extracted later, is not known. Ultimately, soil fractionation followed by PO_4^{3-} determination results in operationally defined chemical "pools" of P, leaving the molecular level detail of soil P cycling unknown.

1.7 Extraction of organic P

Strongly basic or acidic solutions have traditionally been used for the extraction of complex organic matter from soil (Schnitzer & Skinner, 1968). These solutions hydrolyse complex macromolecular soil structures into smaller fractions, as well as releasing them from mineral complexes, thereby solubilising the organic compounds for analysis. The development of an extraction protocol for organic P compounds has followed a similar approach (Mehta *et al.*, 1954). Rather than use sequential acid and base extractions, Bowman & Moir (1983) combined the use of hot (85 °C) NaOH solution with EDTA, at a range of concentrations, in a single step extraction aimed at extracting total organic P from soils. The efficiency of the extractions of soil organic matter were estimated from absorbance at 550 nm and of total P (85 °C) *via* the molybdenum blue test. The combination of NaOH with EDTA extracted twice as much organic P as NaOH alone. The high temperature was found in subsequent studies to be unnecessary and a room temperature NaOH-EDTA extraction has since become the standard method of extraction of organic P from soils for ^{31}P NMR spectroscopy (Cade-Menun & Preston, 1996).

1.8 NMR of organic P

Coinciding with the development of the above extraction methods for organic P, was the introduction of ^{31}P NMR spectroscopy in the early 1980s (Newman & Tate, 1980) for the characterisation of soil P. Using NMR, organic P in soil extracts may be identified to compound class level, for example phosphate monoesters. The method is P-specific and, under the right experimental conditions, is quantitative. The specificity of the method for P is important as



concentrations of P in soil are a fraction of either C or N, with a typical C:N:P ratio of 186:13:1 (Cleveland & Liptzin, 2007), and generally lie below 1 mg kg^{-1} (see Table 1.1). ^{31}P NMR makes it possible to study the P fraction of soils in isolation from the overwhelming carbon content.

Figure 1.10 shows a typical ^{31}P NMR spectrum of a soil extract and identified are the regions in the spectrum corresponding to phosphonates (~ 20 ppm), orthophosphate (~ 6 ppm), orthophosphate monoesters (5 to 2 ppm), orthophosphate diesters (2 to -2 ppm), pyrophosphate (~ -4 ppm), and polyphosphate (~ -20 ppm).

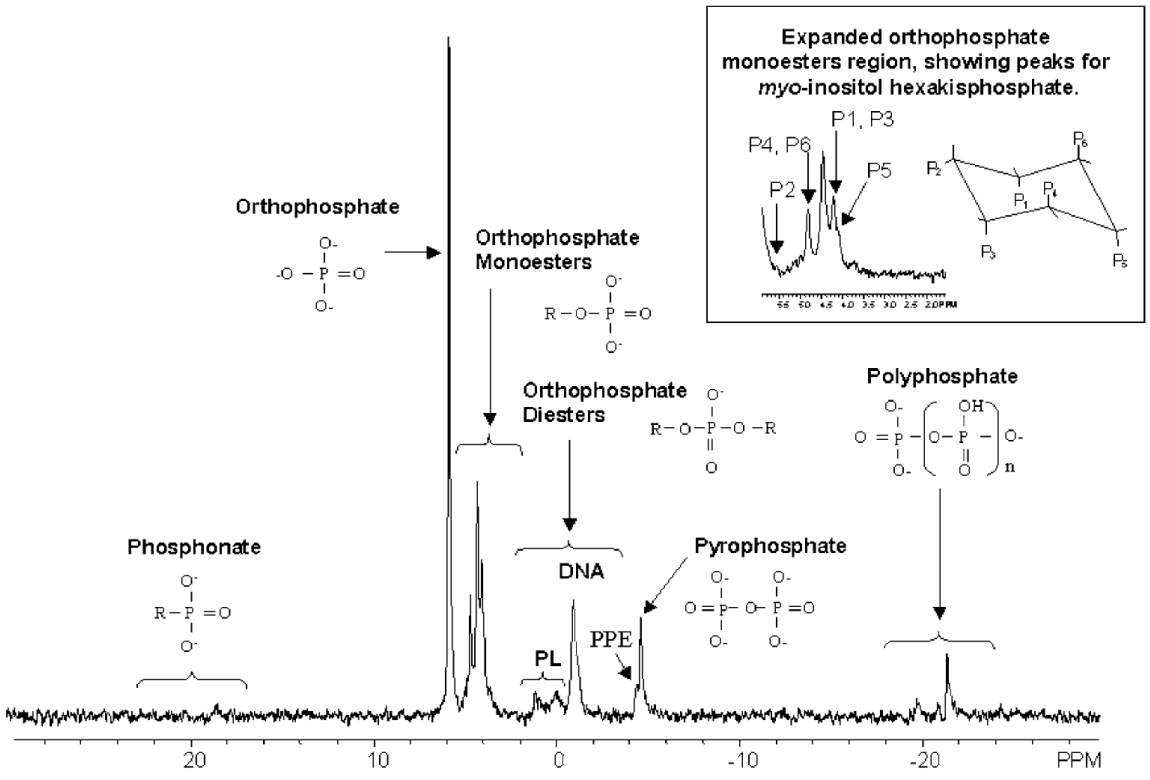
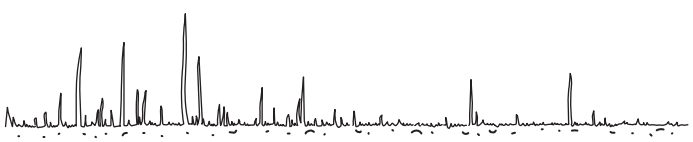


Figure 1.10. A typical ^{31}P NMR spectrum of a soil extracted with NaOH-EDTA. Regions of the spectrum corresponding to P compound class (phosphonate, orthophosphate, orthophosphate monoesters, orthophosphate diester, pyrophosphate, polyphosphate) are indicated. Inset is the expanded monoester region and peaks identified, in this case by spiking, as belonging to IP6. From Cade-Menun (2005).

1.8.1 Identification of individual compounds in NMR

Orthophosphate monoesters are the most abundant P compound class in ^{31}P NMR spectra, which makes for a very crowded monoester region with overlapping peaks. In Figure 1.10, IP6 has been identified by four peaks corresponding to the four chemically equivalent nuclei in the compound (P2; P4, P6; P1, P3; P5). The interpretation of these signals is impacted by the complexity and poor resolution of the spectrum. For example, the P2 peak appears as a slight protrusion from a larger set of overlapping peaks. Underlying signal in the region skewed the peak areas of the IP6 peaks from the expected 1:2:2:1 ratio, with a lot more area under the P5 versus the P2. Some of the contribution to the underlying signal in the monoester region of the ^{31}P NMR spectrum is thought to be due to polymeric-P (P attached to large natural polymeric compounds). However, it is not known just how many P species are responsible for the signal appearing in ^{31}P NMR spectra of soil extracts. Hundreds, or possibly thousands of compounds may be present but while the sensitivity of NMR probes for P remains limited, and the spectrum contains unresolved peaks, the true quantity or variety of P compounds in soil extracts cannot be determined.

Several studies have attempted to overcome the difficulties in assigning peaks to compounds. Turner *et al*. (2003a) published chemical shifts for a range of known soil organic P compounds. The shifts were determined by spiking standards into a soil extract and identification of the peak for each, one at a time. Cade-Menun (2015) made similar observations with a more standardised approach. If pH was the only factor at play, these chemical shifts could have potential for identification of individual compounds in soil extracts, as the pH of soil extracts for NMR are generally >13. However, the ionic strength of a solution also has an impact on the chemical shift of a nucleus, and with so many overlapping peaks, the identification of an individual component in the absence of spiking with a standard and observation of



enhancement of a characteristic resonance (or resonances) is highly questionable (Smernik & Dougherty, 2007).

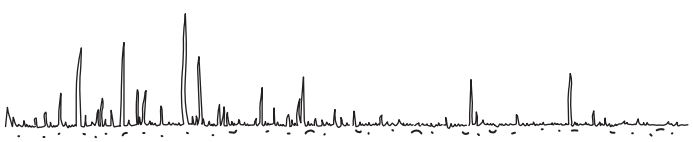
Quantification to compound class level in the strongly basic soil extracts needs to be approached with caution. Makarov *et al*. (2002) questioned the assignment of resonances to phospholipids, arguing that these signals were more likely to be alkaline hydrolised RNA. Therefore, using basic-EDTA extraction and analysis *via* NMR, the concentration of monoesters is likely to be overestimated and that of diesters underestimated. The theory that diesters are more bioavailable and therefore less abundant due to being free in solution and accessible to enzymatic hydrolysis must be treated carefully.

The effect of signal of underlying P nuclei in enhancing that of overlying peaks is well recognised and numerous approaches to obtaining accurate quantification by deconvolution of the spectrum have been attempted (Doolette & Smernik, 2015). IP6 is often the most easily identified compound in a spectrum, and lying within the monoester region, its quantification is greatly affected by the challenges presented by the overcrowded spectrum. The four identified peaks may be integrated to the baseline, as in Turner *et al.* (2003b), however, this may lead to overestimation of the compound, especially where the ratio of the peaks deviates from 1:2:2:1, indicating that one or more signal is enhanced by underlying nuclei. Alternatively, researchers (McLaren *et al.,* 2015; Doolette *et al.,* 2010, 2011a,b) have fitted a broad peak to the monoester region, assumed to be polymeric P. The broad peak is discounted before calculation of quantification.

There are two widely used methods of quantification in ^{31}P NMR of soils. The first is to determine the area of the entire spectrum and to then quantify compounds as fractions of that area and calculate the concentrations on the measured total P of the extract. The other is to use a known quantity of an internal standard, usually methylene diphosphonic acid (MDPA), and

relate the peak areas of compounds to the peak area of the standard. These approaches rely on the signals obtained being fully quantitative, i.e. that the relaxation delay of the experiment exceeds the T_1 of all relevant nuclei. Determination of T_1 values is lengthy and frequently omitted, with reliance on published T_1 values (McDowell *et al*., 2006). The relaxation delay may be influenced by sample properties, for example paramagnetic ions bound to compounds of interest shorten T_1 times. While relaxation delays < 5 s were common in early studies (Cade-Menun, 2005), more recent work use delays of 40 s and longer in acknowledgement of the improved accuracy of quantification at longer relaxation delays (McLaren *et al*., 2017).

While ^{31}P NMR may be P-specific, there are technical challenges in terms of identification and quantification as discussed here. The sensitivity of the instrumentation is also an important issue, with each experiment requiring a minimum of 12 h of acquisition for good signal-to-noise ratio. To improve the signal-to-noise ratio by a factor of two, the acquisition time must be squared. This means that significant improvements in sensitivity are unfeasible and costly. Even with higher sensitivity, new P compounds cannot be identified using ^{31}P NMR, and the technology is limited in providing information about the chemical structure of compounds at distance from the P nucleus. Previous 2-dimensional studies have faced the same challenges in terms of probe sensitivity and complexity of extracts and have required 40 h of acquisition time for spectra of extracts spiked with IP6 (Vestergren *et al.,* 2012). ^{31}P NMR spectroscopy of NaOH-EDTA extracts has brought about great progress in our understanding of the composition of the organic P compounds in soils and other matrices, however, the current state of analysis is at the limit of what NMR is capable of, and further advances will not be possible without remarkable improvement in the technology.



1.9 Chromatography of organic P

Before the advance of ^{31}P NMR, analysis of organic P compounds in extracts was *via* chromatography. P compounds do not have a specific UV/Vis absorbance wavelength, and compounds such as IPxs have no chromophore whatsoever. In the absence of direct detection methods, such as MS, detection has taken varied forms. In the 1950s, Anderson (1955) demonstrated paper chromatography of IPxs, using a molybdate-perchloric reagent spray developed by Hanes and Isherwood (1949) to detect the PO_4^{3-} released from the IPx. Early work by Cosgrove (1962, 1964) and Cosgrove & Tate (1963) relied on dephosphorylation of IPx to their inositol backbone in order to identify stereoisomers of IPx in soil *via* thin layer chromatography (TLC) and gas chromatography (GC). GC of P compounds requires derivatisation of the phosphate moiety with a non-polar group such as trimethylsilyl. This approach requires multiple derivatisation and clean-up steps and results are variable. The derivatisation of sterically hindered and highly polar compounds such as IPxs for GC analysis is impractical.

Chromatographic methods were superseded by ^{31}P NMR in the early 1980s, but the increasing sophistication of HPLC instruments has continued to offer alternative opportunities for analysis. Again, challenges are faced due to the low concentrations of P compounds found in soil extracts, and the wide range of chemical properties of organic P compounds from phospholipids to inositol phosphates. However, chromatographic analysis is much faster than NMR, has the potential to separate individual species, and may be combined with MS for compound identification. Reverse-phase HPLC offers limited options for separation of P compounds in strongly basic soil extracts, as they are too polar to be retained on non-polar stationary phases. Therefore, HPLC usually takes the form of normal phase chromatography using silica-based stationary phases for separation of intact phospholipids, sometimes ion-pair

chromatography, and more frequently ion exchange systems are used. GC is more commonly used for the analysis of fatty acids hydrolysed from phospholipids. Ion exchange chromatography is commonplace in the analysis of P compounds, particularly IPxs, in food extracts and its use has become more prevalent in soil, manure and sediment research (Ray *et al.,* 2012, Waithasong *et al.,* 2015, Sjöberg *et al.,* 2016). Combining chromatography with reference materials, individual compounds can be identified by retention time and quantified by peak area and standard addition.

1.9.1 Detection of P compounds

One of the methods of detection in paper chromatography of P compounds was with an $FeCl_3$ sulphosalycylic acid spray known as Wade's reagent (Wade & Morgan, 1955). This has been incorporated into HPLC with post-column derivatisation of P compounds with ferric chloride sulphosalycylic acid followed by UV detection at 500 nm (Bos *et al.*, 1991, Rounds & Nielsen, 1993). The quantification is on the basis of the reduction in concentration of the Wade's reagent in the eluent solution after complexation with the phosphate group of analytes. Modern ion chromatographs incorporate electrochemical ion detection methods, such as conductivity or amperometry, which do not require derivatisation of analytes post-column. ICP-AES is also used as a P-specific detection method (de Brabandere *et al.,* 2008).

1.10 Mass spectrometry of organic P compounds

Prior to the development of soft ionisation methods in the 1980s, compounds were submitted mostly to electron ionisation and identified based on the M^+ and fragment ions in their mass spectra. This required isolated compounds to be introduced in the ion source to prevent overlapping fragment patterns causing confusion. This could be achieved either by chromatographic purification of the compounds for solid probe introduction or the use of gas chromatography (GC) to resolve mixtures on-line in GC/MS. Phosphorylated compounds

would generally have been hydrolysed, enzymatically or chemically, derivatised and the organic moieties determined by GC/MS (Myher and Kuksis, 1982). With the advent of soft ionisation methods, such fast atom bombardment MS (FABMS) or liquid secondary ion MS (LSIMS), the analytical utility of MS was greatly extended to include polar analytes, including phosphorylated and sulphated organic compounds (Dallinga *et al*., 1989).

The development of electrospray and atmospheric pressure ionisation in MS offered much more versatility in the "soft ionisation" of compounds of widely varying polarity and molecular weight (Fenn *et al*., 1989; Dickson *et al*., 2009). ESI in particular general yields very simple mass spectra dominated by $[M+H]^+$ or $[M-H]^-$ ions depending on the ionisation mode selected. It is this property that has seen the technique becoming routinely used in the identification of compounds in complex mixtures and the wide application of ESI-HRMS in the -omics (proteomics, metabolomics, etc.) fields (Yates *et al.,* 2009). Briefly, ESI involves the nebulization of the analyte solution under the influence of a strong electric field, producing a fine spray of highly charged droplets. It is theorised that the droplets diminish in size across the electric field until the solvent is either completely evaporated and the charge is conferred to the analyte, or the charged ion is desorbed from the solution due to a high surface charge density on the shrinking droplet. The result is vaporised ions entering the capillary tube and transferred *via* the electronic optics of the mass spectrometer.

Mass spectrometric characterisation of P-containing compounds is now extensively used in biochemical analyses, for example in the detection of phosphorylation sites on proteins (Mann *et al.,* 2002), or in the characterisation of the phospholipid profile of discrete biological systems (Wang *et al*., 2005), and in the study of DNA and the identification of methylation patterns (Ehrich *et al.,* 2005). Mass spectrometry enables direct detection of ions and individual molecular species targeted through experiments like tandem mass spectrometry (MS/MS),

selected ion monitoring (SIM) for sensitive detection of low abundance compounds, or multiple reaction monitoring (MRM), which identifies specified fragmentation patterns. The prevalence of the phosphate moiety in organic P compounds is often exploited in these studies, for example phosphorylated peptides are identified by the neutral loss of (HPO_3) in MS/MS experiments.

However, the development of mass spectrometry methods for the characterisation of organic P compounds in environmental matrices is still in its infancy and MS analysis of P compounds has been approached in just a small selection of studies. El Rifai *et al.* (2008) combined negative ion SIM at *m/z* 659 and 79 with size exclusion chromatography (SEC) to determine the presence of IP6 at low abundance in extracts of wetlands soils. De Brabandere *et al.* (2008) used LC coupled to ICP-AES and ESI-MS/MS to identify P compounds in modified aquatic sediment extracts. ICP-AES was used to screen the chromatogram for P-containing compounds. A number of nucleotides were identified by an enhanced product ion scan on a quadrupole ion trap mass spectrometer. A precursor ion scan was used to produce an ion chromatogram of the ions containing PO_3^- or $H_2PO_4^-$ in much the same way as phosphorylated peptides are identified. Sjöberg *et al*. (2016) later combined IC with MRM for the identification of IPxs in lake sediments and this study is discussed further in Chapter 3. The high-resolution of Fourier-transform ion cyclotron resonance (FTICR) MS was demonstrated by Cooper *et al*. (2005) for the identification of unknown P compounds in fulvic acid mixtures and wetlands. However, this method is heavily reliant on accurate mass detection and assignment of molecular formulae. Identifications in FTICR require validation *via* chromatography, reference standards or further MS experiments. The challenges identified in soil MS studies are the high ionic strength of the extract solutions, which are not well suited to HPLC analyses, and the vast number of compounds in the extracts requiring high mass and chromatographic resolving powers for their separation and characterisation.

1.10.1 High resolution mass spectrometry

HRMS is becoming more and more prevalent and is achieved using either an FTICR-MS instrument, or more commonly, an Orbitrap instrument. The Thermo Fisher Orbitrap Elite ™ mass spectrometer contains an octopole linear trap which scans ions quickly at low resolution. Ions are then transferred to the Orbitrap detector which captures ions in an electric field and causes them to orbit inside the detector in a path determined by the mass-to-charge ratio of the ion. The electric signal emitted by the orbiting ion is then Fourier transformed to generate a mass spectrum. The Orbitrap can accumulate ions in the trap, increasing the signal-to-noise ratio and therefore the resolution of the mass spectrum.

This high resolution enables ions with close *m/z* values to be distinguished from each other. Figure 1.12 demonstrates the potential of applying HRMS to highly chemically complex extracts of river water, resolving numerous ions within a 0.3 *m/z* window. HRMS also offers high accuracy of *m/z* detection (± 5 ppm of the theoretical monoisotopic *m/z*). The use of ESI and high resolution combined with high accuracy mass spectrometry is essential in the detection and identification of individual ions in complex mixtures, leading to ESI-HRMS becoming an invaluable tool in the fields of proteomics and metabolomics. High throughput methods for the analysis of proteins, and the phosphorylation sites of proteins have become standard in biochemical research, broadening our understanding of important biochemical processes.

The same can, and must, be achieved in the field of DOM research. The challenge ahead lies in the complexity and variety of environmental matrices. By comparison to soil extracts, biological systems are well-defined matrices, but extracts of soil vary in composition between soil type, inputs – both natural and anthropogenic – mineral content, pH and even depth. Variation between matrices such as sediments, manures, wastewater treatment effluent are even

greater. A lot of investment in development is required to produce methods of analysis for these matrices, so that we can develop our understanding of molecular level mechanisms as has already been done for biochemical systems. The combination of chromatographic systems with ESI-HRMS is the technology which offers a real chance of discovering the molecular level nature of the P biogeochemical cycle. This is surely the means for informing active management of human impact on the environment.

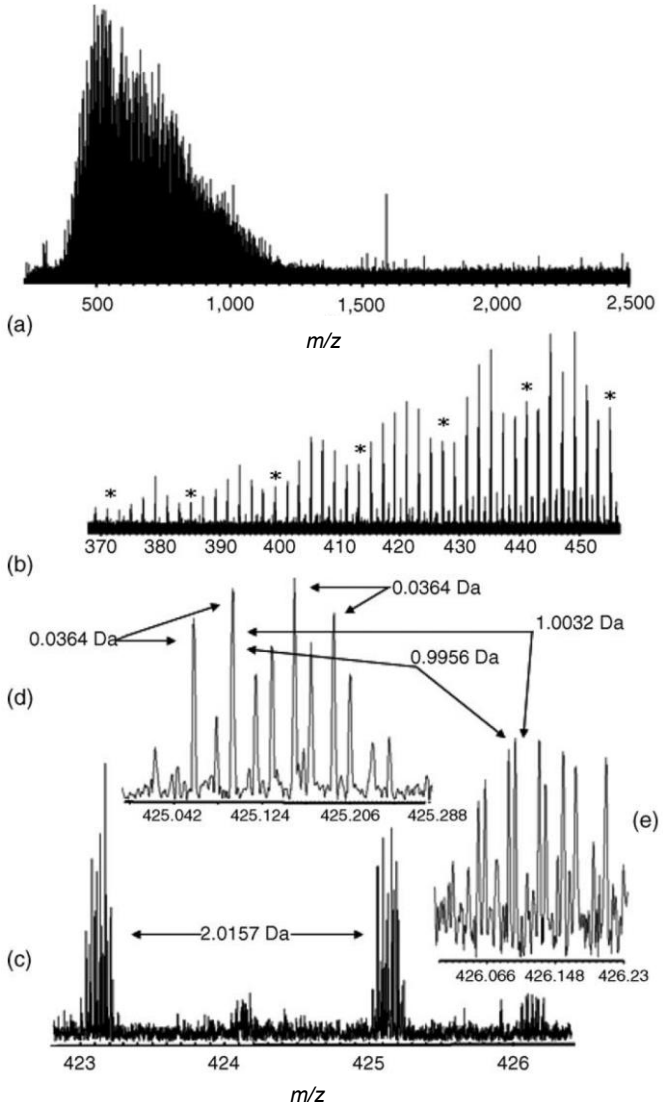


Figure 1.11. Positive mode ESI-FTICR-MS mass spectra of Suwanee River fulvic acid mixture. (a) The entire spectrum from *m/z* 225 to 3000 demonstrating the complexity of natural organic matter samples. (b) and (c) highlight the *m/z* 370 to 450 and *m/z* 423 to 426 regions, respectively, while (d) and (e) display *m/z* 0.3 windows and indicate spacing between ions.

These figures show the power of HRMS to handle complex samples and resolve ions in close proximity to each other. From Cooper *et al*. (2005).

1.11 Aims

The current landscape in organic P research suggests that the characterisation of P in environmental matrices is on the brink of a major development. NMR spectroscopy has shed light on biologically-derived P compounds, and hinted at a large pool of, as yet, uncharacterised monoesters but has reached the limit of its power to resolve individual compounds. Meanwhile, the technological approaches to molecular analysis in chromatography and particularly ESI-HRMS have advanced considerably in other fields. The aims of this thesis were developed, in light of this potential of analytical chemistry, to derive further molecular understanding of the P biogeochemical cycle through the use of analytical methods capable of fully resolving and unambiguously characterising and quantifying organic P at the molecular level.

Overall aims were:

1. Develop methods of analysis of organic P compounds in complex environmental matrices, using IP6 as a model compound.
2. Apply the method to investigations of the molecular behaviour of IP6 in soils.

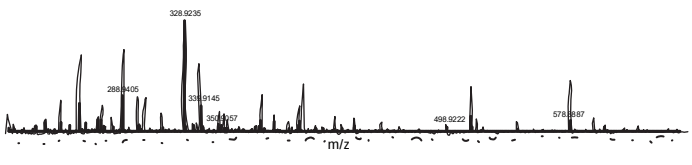
Specific objectives were:

1. Characterise the ESI-HRMS analysis of IP6 and IP5.
2. Demonstrate the combination of IC with ESI-HRMS for analysis of IP6 as a reference solution, and isolated from a soil extract matrix.
3. Apply IC analysis of IP6 in multiple matrices (soils and manures).
4. Validate the quantification of IP6 in IC against current ^{31}P NMR spectroscopy methods.
5. Determine the extraction efficiency of NaOH-EDTA extraction for IP6 from a range of soils and identify soil characteristics contributing to the extraction efficiency.

6. Compare the extraction efficiency of IP6 to that of total P from soils using NaOH-EDTA.
7. Investigate the behaviour of IP6 in soil from a commercial reference solution and a natural source, poultry litter, over the course of an incubation experiment.
8. Investigate the mobilisation of IP6 from poultry litter applied to soil and incubated for 1 day and 14 days.

1.12 Thesis overview

These aims will be addressed in the following chapters. The methods and materials used are described in Chapter 2, providing the outline for the combination of IC with ESI-HRMS for analysis of IP6 from soils. The extraction methods and soil experiments are also described in detail. In Chapter 3 the behaviour of IP6 and IP5 in ESI-HRMS is reported. The observed fragmentation patterns are confirmed using MS/MS experiments, and investigations of impurities in the IP6 reference standard using ESI-HRMS characterisation of IC eluate fractions are described. The application of these methods coupled with insight into behaviour of IP6 in ESI-HRMS are then applied to the identification and quantification of IP6 in soil and manure extracts in Chapter 4. The analysis method is compared with ^{31}P NMR spectroscopy in terms of quantification and identification. The relationship between extraction efficiency of NaOH-EDTA for IP6 from soils and the properties of the soils is explored in Chapter 5. IP6 is quantified from spiked and control soil extracts of different type and land cover and the extraction efficiency determined along with metal concentrations, organic content, pH and total P of the soils. The correlations between IP6 extraction efficiency and soil characteristics are discussed. The fate of IP6 from a natural source, in the form of poultry litter applied to soil, is investigated in Chapter 6 through a soil incubation experiment and a transport experiment.



Chapter 7 describes a series of pilot experiments to investigate the use of IC and ESI-HRMS for the analysis of a range of other organic P compounds. Further studies and research questions arising from this work are proposed and the research findings are summarised.

Chapter 2. Materials and methods

2.1 Materials

Extraction chemicals $Na_2EDTA.2H_2O$ and NaOH were purchased from Sigma Aldrich (UK), and Thermo Fisher Scientific (Hemel Hempstead, UK), respectively.

Reference standards IP6 (*myo*-inositol 1,2,3,4,5,6 - hexakisphosphate sodium salt, $Na_{12}C_6H_{12}O_6(HPO_3)_6$) and IP5 (*myo*-inositol 1,3,4,5,6 - pentakisphosphate pentapotassium salt, $K_5C_6H_{12}O_6(HPO_3)_5$) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Methylene disphosphonic acid (MDPA) was purchased from Sigma Aldrich (UK).

Glassware was washed with Decon 90, acid (5 % HNO_3) washed, rinsed with double distilled water (DDW) and furnaced at 450 °C for 4 h.

IC vials were acid washed and rinsed × 6 with DDW.

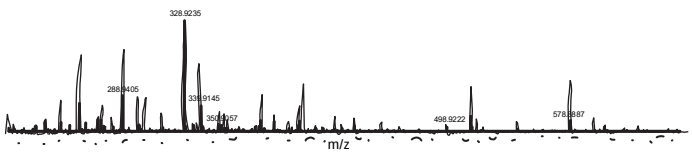
2.2 Soil preparation for extraction

Soils for Chapters 4 and 5 were collected from the upper 10 cm after removal of grass and litter. They were then air dried at ambient temperatures to constant mass and sieved to 2 mm.

Soil for the incubation and transport experiments were BS3882:2015 certified topsoil purchased from Raycox Turf Ltd. (Bristol) and accompanied by a declaration of compliance and analysis results.

2.3 NaOH-EDTA Extraction

The extraction method for soil organic P was based on Turner (2008). A solution (30 mL) of 0.25 M NaOH/ 0.05 M Na_2EDTA was added to soil (1.5 ± 0.01 g) in a 50 ml centrifuge tube. Tubes were shaken horizontally at 240 rpm at room temperature for 4 h, before centrifugation at 3300 rpm for 45 min.



2.4 Extract preparation for analysis

The supernatant was decanted (20 mL) to a 28 mL vial for NMR analysis. The internal standard (1.02 mL 50 µg $P.mL^{-1}$) methylene diphosphonic acid (MDPA) was added to the 20 mL aliquot for NMR which was then freeze-dried. A 1 mL aliquot of extract was filtered through a 0.2 µm PTFE syringe filter to an IC vial for IC analysis. The remaining supernatant was retained for total NaOH-EDTA extracted P analysis. IC and TP aliquots were stored at –20 °C until analysis.

2.5 Soil spiking with IP6

For extraction efficiency experiments, 1.5 g air-dried soil samples were mixed with either 1.04 mL DDW or 616 mg L^{-1} IP6 stock solution. Soils were stirred to completely homogenise the mixture and incubated for 2 h at room temperature before extraction as per 2.3.

2.5.1 Extraction efficiency calculations

Extraction efficiency was calculated by combination of the concentration of IP6 added to the soil ([A]) to each control soil IP6 concentration ([C]) to obtain a value for the theoretical IP6 concentration ([A] + [C] = [T]) for that soil. This value ([T]) would represent the soil IP6 concentration, assuming 100 % extraction efficiency. The calculated extraction efficiency is then the spiked soil IP6 concentration ([S]) expressed as a percentage of the theoretical IP6 concentration.

$$\frac{[S]}{[T]} x\ 100 = EEIP6$$

The extraction efficiency for total P was determined by expressing the NaOH-EDTA extracted P concentration as a percentage of bulk soil total P concentration.

2.6 Soil dry matter content

Soil dry matter content (DMC) and moisture content (MC) was determined by weighing field moist soil (10 g) into a dry and pre-weighed Petri dish in triplicate. The soil was dried for 24 h in an oven at 105 °C, allowed to cool in a desiccator, then re-weighed. DMC and MC were calculated as percentages of field moist soil.

2.7 Water holding capacity

Soil water holding capacity (WHC) was determined volumetrically. Glass wool (0.25 g) was firmly rolled into a cylinder and placed in the neck of a glass funnel which had a length of rubber tubing sealed closed with a clip attached to the bottom. DDW (10 mL) was added to the funnel and left for 30 min. The tube was unsealed, and the water was left to drip to a clean, dry measuring cylinder for 30 min. The volume collected was recorded. This was repeated twice more to determine volume of water retained by the glass wool. The procedure was then repeated with 10 g fresh soil added to the funnel before addition of DDW, again in triplicate. The volume of water collected in the cylinder was recorded.

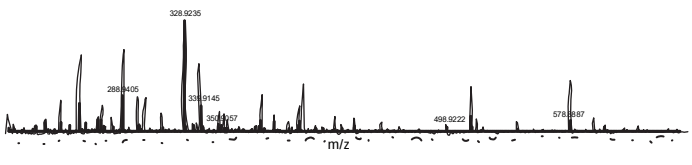
WHC was calculated using the following equations:

Equation 2.1

$$10 - (volume\ retained\ by\ glass\ wool\ +\ volume\ collected\ in\ cylinder) = volume\ DDW\ retained\ by\ soil\ (A\ mL)$$

Equation 2.2

$$(10\ x\ A\ mL) + MC = WHC\ mL\ per\ 10\ g\ fresh\ soil$$



2.8 Soil, poultry litter, and IP6 incubation set up

Soil incubations were prepared after Charteris (2016) in glass tubes measuring 10 cm high x 2 cm diameter, with one end of the tube tapered to a small hole (~ 5mm) at the base (Figure 2.1). The hole was plugged with a portion of furnaced glass wool and the mass of the tube and glass wool was recorded. Air dried soil sieved to 2 mm (10 g) was added to each tube and gently compacted. The soil was then rewetted to 60 % WHC by mass with DDW, covered with pierced foil to allow oxygen in, but minimise water loss, and pre-incubated in the dark at 17 °C for 3 weeks prior to treatment. Every week throughout the pre-incubation and incubation period, each tube was weighed and re-wetted to 60 % WHC by mass using DDW from a spray bottle. The water loss since the previous week was recorded to determine the evaporation rate.

The incubation treatments were: soil control (CTRL), soil with poultry litter added (PL) and soil with commercial IP6 compound solution (PS). Each treatment had three replicate tubes for each sampling timepoint.

At the beginning of Week 0, poultry litter (0.17 g DW) was added to the PL soils. The application rate (10.86 t PL ha^{-1}) was based on DEFRA guidelines for upper N application (250 kg N ha^{-1} yr^{-1}) and calculated on typical N concentrations (23.02 kg N t^{-1}) of poultry litter. IP6 standard solution (0.25 mL of 3.3 mg IP6 mL^{-1}) was added to the PS soils by injection with a syringe which was inserted to 1 cm from the bottom of the soil and the plunger depressed as the syringe was withdrawn, ensuring even coverage. The IP6 concentration of the PS solution was intended to be the same per 10 g soil as that added in the poultry litter treatment. This was based on initial IP6 concentration determination of the poultry litter. All soils were re-wetted to 60 % WHC. Week 0 tubes were sampled 2 h after treatment.

2.8.1 Sampling

Homogenisation of the 10 g soil sample was achieved by turning over with a spatula. An aliquot (1.5 g) was taken for immediate NaOH-EDTA extraction as per section 2.3. The moisture content of the soil was taken into account when calculating the IP6 concentration. This sampling procedure was repeated for Weeks 1, 2, 3, 5, 7, 9 and 11.

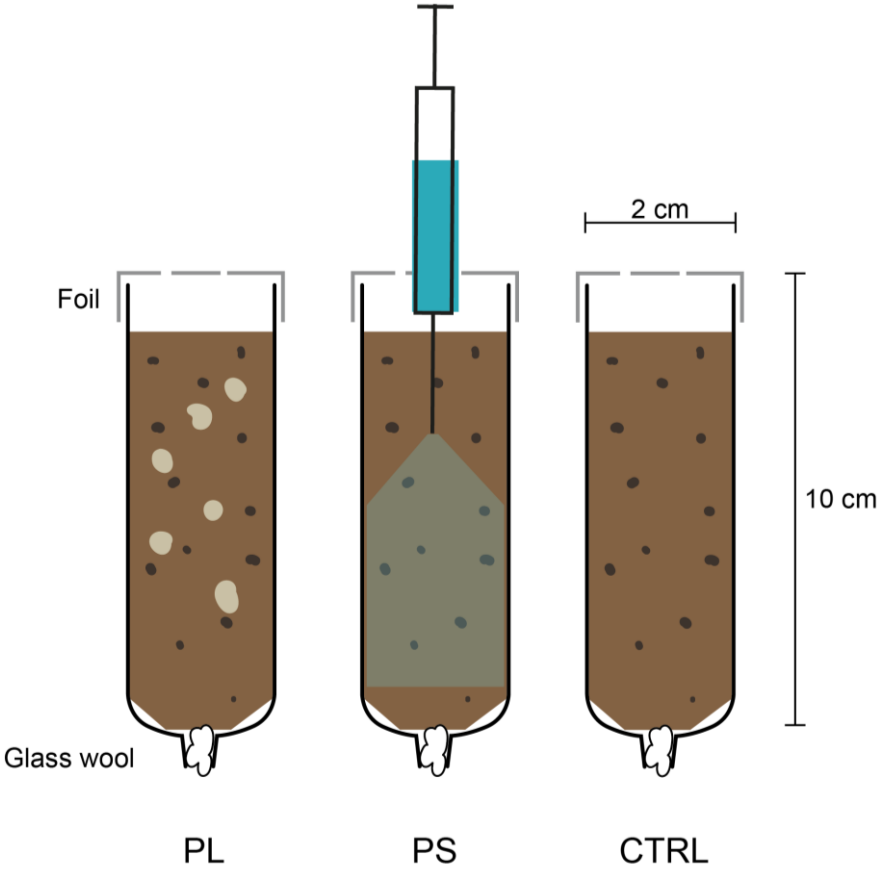


Figure 2.1. Set up of tubes for soil incubation experiment. Treatments are PL: poultry litter, PS: phytate (IP6) solution, CTRL: control (no treatment).

2.9 Lysimeter set up

Glass lysimeter columns were 25 cm high x 10 cm diameter and tapered to a small (~ 1 cm) hole at the base (see Figure 2.2). Each column was fitted with a wire mesh which supported approximately 1 cm depth of washed 10 mm gravel to allow free drainage of water from the

column. The column was marked in 5 cm intervals to 20 cm above the level of gravel, and a final mark at 22 cm. Fresh soil, sieved to 6 mm, was added to ~ 1 cm above each 5 cm mark and gently compressed back to the mark. At the top of the column, the soil was added to the 22 cm mark, before being gently compressed to 20 cm. The soil moisture content was measured before packing of each column using an electronic soil moisture probe. The mass of soil in each column was recorded and DDW was added to each column to 60 % WHC by mass. The columns were covered over with foil to minimise water loss and pre-incubated in the dark at 17 °C for 5 days prior to treatment.

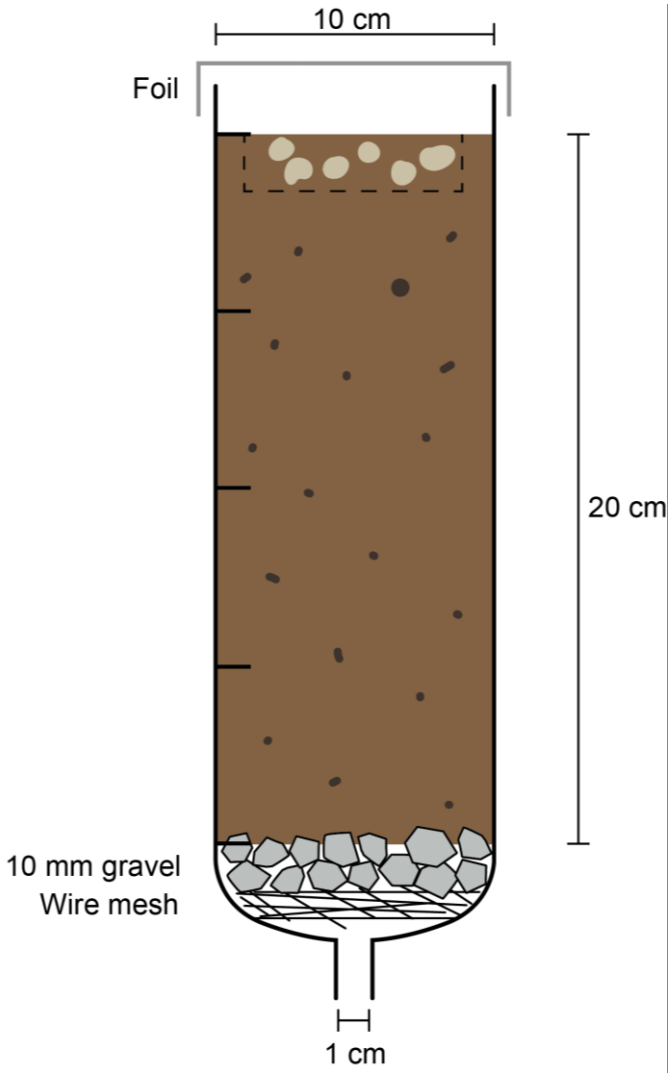


Figure 2.2. Lysimeter column set up. Dotted line indicates soil removed for mixture with poultry litter for the 14-day and 1-day poultry litter treatments.

The treatments were control (CTRL), poultry litter with 14-day incubation (PL-14D) and poultry litter with 1-day incubation (PL-1D). Each treatment had 3 replicate columns. The columns were re-wetted each week to 60 % WHC by calculation of the rate of water loss per unit area based on the incubation experiment.

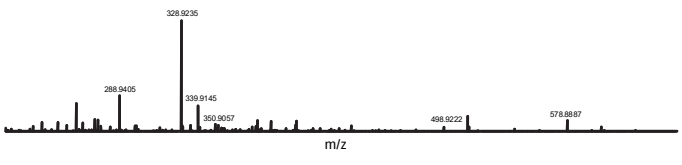
On day 0 of the experiment, an 8 cm diameter x 2 cm depth portion of soil was removed from the centre of the PL-14D columns (Figure 2.2) to a clean beaker. Poultry litter (5.45 g) was thoroughly mixed with the soil and the mixture was returned to the top of the soil column and gently tamped down. The application rate was the same as that for the incubation experiment. All columns were then re-wetted to 60 % WHC as before. On day 13 of the experiment, poultry litter was added to the PL-1D columns in the same way.

2.9.1 Leaching

The columns were leached on day 14 after re-wetting to 60 % WHC. The leaching procedure informed by Lloyd *et al.* (2012) was to gently pour 50 mL DDW to the top of each soil column over 10 min to simulate a 38 mm/hr rainfall rate, typical in storm events. The DDW was added in 50 mL aliquots until breakthrough discharge was achieved. Twenty-minute intervals were then introduced between 50 mL aliquots, and leachate discharge per 50 mL was measured. Leaching continued until leachate discharge was constant for each 50 mL DDW "rainfall event".

2.9.2 Soil core sampling

Soil cores were taken from each column at 3 depths: 0 – 5 cm, 5 – 10 cm and 10 – 20 cm. Cores were taken one depth at a time with a stainless-steel metal tube (2 cm diameter), which was gently pushed and twisted into the soil, then twisted back out with the soil core intact. The core was then pushed through the tube to a foil container. The mass of the core at each depth was recorded. The soil was homogenised by turning over with a spatula. An aliquot (1.5 g) was



taken for NaOH-EDTA extraction, and a 1 g aliquot taken for pH measurement. The 1.5 g aliquots were frozen until extraction as per 2.3.

2.10 Analytical methodology

2.10.1 Ion chromatography

Ion chromatography was performed on a Dionex ICS-5000 (Thermo Scientific, Hemel Hempstead, UK) equipped with a KOH eluent generator, ion suppressor and conductivity detector. Compounds were separated on an Ionpac AS11 column (2 x 250 mm; Thermo Scientific) preceded by an AS11G guard column (2 x 50 mm). The flow rate was 0.250 mL min^{-1}, and column oven temperature was 30 °C. The elution gradient comprised an initial 10 min equilibration at 4 mM KOH, followed by 0 to 19 min: 4 mM KOH, 19 to 24 min: ramp to 70 mM, 24 to 29 min: 70 mM KOH, 29 to 30 mM: ramp to 4 mM KOH.

A cleaning gradient was set up as follows: 0 min: 4 mM KOH, 1 min ramp to 95 mM KOH, 1 to 41 min: 95 mM, 42 to 60 mM: 95 mM to 4 mM KOH. The cleaning gradient was run between all samples to prevent carryover of IP6 in the system. The parameters for the cleaning gradient were determined by injecting an IP6 standard (1 μM), followed by the cleaning gradient and a Milli Q blank afterward, then increasing the duration of the high concentration KOH until there was no IP6 detectable in the Milli Q blank.

Chromatograms were analysed in Chromeleon 7.0 (Thermo Scientific).

2.10.1.1 Preparatory IC

Eluate fractions were collected in furnaced amber glass vials post-detection at defined intervals for HRMS analysis.

2.10.1.2 Calibration curve

A calibration curve for IP6 was prepared using stock solutions at 0.005, 0.01, 0.05, 0.1, 0.5 and 1 µM concentrations of the standard compound. The lowest concentration standard (0.05 µM) was analysed ten times and the LOD and LOQ were calculated according to International Conference on Harmonization Guidelines (ICH, 2005) using the standard deviation (σ) of the area of the IP6 peak from the ten injections, and the slope (m) of the calibration according to the formulae:

$$LOD = \frac{3.3\ x\ \sigma}{m}$$

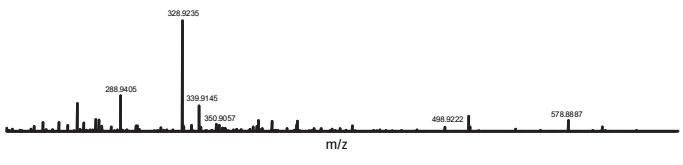
$$LOQ = \frac{10\ x\ \sigma}{m}$$

2.10.1.3 Standard addition

Quantification of IP6 in soil and manure extracts was *via* standard addition. Soil extracts, cow manure and sheep manure extracts were diluted 20-fold with Milli Q water. Pig manure and chicken manure extracts were diluted 50-fold. The concentration of IP6 standard to be added was calculated after initial screening of the diluted extracts and quantification using the calibration curve. Three standard additions were made and the concentration of IP6 in the unadulterated extract was calculated by preparation of a standard addition plot, according to the equation:

$$[IP6] = c/m$$

Where c is the intercept, and m is the slope of the fitted line.



2.10.2 ESI-HRMS

High-resolution mass spectrometry analysis was performed on an Orbitrap Elite™ mass spectrometer (Thermo Scientific) with an ESI source. The Orbitrap was operated in negative ion mode, calibrated using negative ion calibration solution (Thermo Scientific), and tuned automatically on the *m/z* 328.9 (IP6 $[M-2H]^{2-}$). Mass spectra were analysed using Xcalibur 3.0.63 (Thermo Scientific).

2.10.2.1 Sample preparation for HRMS

Reference (IP6 and IP5) solutions were prepared at 20 ppm in DDW. Preparatory IC eluate was analysed directly.

2.10.2.2 Direct infusion

Solutions were directly infused at 10 µL min^{-1} for acquisition of full mass spectra, and at 6 µL min^{-1} for MS/MS analysis. The source voltage was −1.8 kV, sheath gas (nitrogen) flow rate was 30 arbitrary units (arb), auxiliary gas (nitrogen) flow rate was 0 arb and the sweep gas (nitrogen) flow rate was 1 arb. The capillary temperature was set to 275 °C. Full mass spectra were recorded at 120,000 resolution and 50 scans were averaged to increase the signal-to-noise ratio.

2.10.2.3 MS/MS

Fragmentation was *via* higher energy collisional dissociation (HCD) at 65 % normalised energy. MS/MS spectra were recorded at 15,000 resolution to all a higher scan rate which would be useful for future LC/MS. MS/MS spectra were collected for the 20 most abundant ions in the spectrum.

2.10.3 NMR

2.10.3.1 Sample preparation for ^{31}P NMR

Freeze-dried soil extracts were prepared for NMR by dissolving 100 or 200 mg homogenised freeze-dried powder in 0.9 mL 1 M NaOH/ 0.1 M Na_2EDTA solution and 0.1 mL D_2O. Re-dissolved extracts were centrifuged at 6000 rpm for 20 min and the supernatant decanted to a 5 mm NMR tube for immediate analysis.

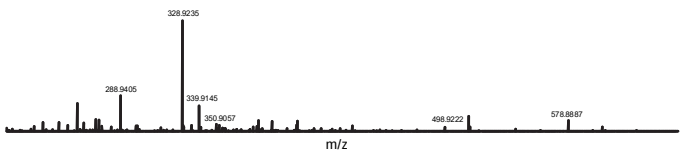
An equimolar MDPA:IP6 solution was prepared and run under the same conditions as the soil extracts. A response factor was calculated for each of the four IP6 resonances and used to quantify the compound in the soil extracts.

2.10.3.2 Acquisition of NMR spectra

NMR spectra were acquired on a 500 MHz Varian VNMR S500 equipped with an Agilent OneNMR probe. Parameters were 45° pulse, 0.66 s acquisition time, 3.0 s relaxation delay, 16000 scans at 25 °C with proton decoupling.

2.10.3.3 NMR analysis

NMR spectra were analysed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). The PO_4^{3-} peak was set to 6 ppm. Control and spiked spectra for the same soil extract types were overlaid and IP6 peaks were identified by the increase in peak height of the four peaks (IP6a, IP6b, IP6c, IP6d). Baseline correction was *via* Whittaker Smoother. Internal standard MDPA, and the four peaks were integrated, and the integral values were corrected using the predetermined response factor for MDPA versus IP6 under experiment conditions. Quantification of IP6 was based on the lowest corrected peak area as this was deemed to be least influenced by underlying signal from other ^{31}P nuclei in the extract.



2.10.4 Total P of soil extracts – digestion and analysis

Soil extracts were digested using a persulfate microwave digestion based on Johnes & Heathwaite (1992). Phosphate concentrations were determined colorimetrically, measuring absorbance at 880 nm on a Skalar Continuous Flow analyser (Skalar San ++, the Netherlands).

2.10.5 Bulk soil metal and P determination

2.10.6 Soil digestion

Soil samples were prepared according to the UK Environmental Agency guidelines (UK Environmental Agency, 2006). Air dried soils were first sieved to 2 mm and material, other than stones, was ground to pass through the sieve. The process was repeated using a 300 µm sieve. A 1.0 g aliquot of the 300 µm soil was added to a 100 mL conical flask with 7 mL concentrated (16 M) HNO_3 and 21 mL concentrated (12 M) HCl. The conical flask was covered with a clock glass and the mixture was gently refluxed on a hot plate for 5 h. The flask was removed from the hot plate and 50 mL DDW was added. The solution was returned to the hot plate and brought just to boiling point. The solution was allowed to cool to room temperature before being quantitatively transferred to a 100 mL volumetric flask. The solution was made up to the 100 mL mark with DDW. The solution was then filtered through a hardened, ashless Whatman Grade 540 Quantitative filter paper.

2.10.6.1 Inorganic Analysis

Determination of Al, Ca, Fe, Mg, Mn and P concentrations in soils after aqua regia digestion, was *via* ICP-OES using an Agilent series 710 (Agilent Technologies LDA, Cheshire, UK).

2.10.7 Loss on ignition (LOI)

Organic content (LOI) was determined after Wright *et al.* (2008). Air dried soil (~ 500 mg) was weighed into a pre-weighed 3 mL vial. Soils were dried overnight at 105 °C, allowed to

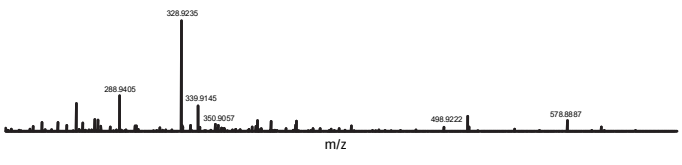
cool in a desiccator and weighed (A). The vials were then put in a furnace at 550 °C for 4 h, allowed to cool in a desiccator and weighed again (B).

LOI was calculated according to the following equation:

$$LOI\ (\%) = \frac{A - B}{A} x\ 100$$

2.10.8 pH

Soil pH was determined in 1:2.5 soil mass to DDW ratio. DDW (2.5 mL) was added to 1 g soil and left to equilibrate for 1 h before determination with a calibrated pH probe.



Chapter 3. High-resolution mass spectrometric analysis of *myo*-inositol hexakisphosphate using electrospray ionisation Orbitrap

3.1 Abstract

The phosphorus storage compound in grains, IP6, is important for nutrition and human health, and is the most abundant organic phosphorus compound in soils. Methods for its determination have traditionally relied on complexation with iron and precipitation, acid digestion and measurement of phosphate concentration, or ^{31}P NMR spectroscopy. Direct determination of phytic acid (and its homologues) using mass spectrometry has, as yet, found limited application to environmental or other complex matrices. The behaviour of phytic acid in electrospray ionisation high resolution mass spectrometry (ESI-HRMS) and its fragmentation, both in-source and *via* collision induced dissociation have not been studied so far. The negative ion mass spectrometry and MS/MS of IP6, and the lower inositol pentakisphosphate (IP5), using an ESI-Orbitrap mass spectrometer is described. The purity of the compounds was investigated using anion exchange chromatography. IP6 is highly anionic, producing multiply charged ions and sodium adduct ions which readily undergo dissociation in the electrospray ion source. MS/MS analysis of the phytic acid $[M-2H]^{2-}$ ion and fragment ions and comparison to the full MS of the *myo*-inositol pentakisphosphate reference standard, and the MS/MS of the pentakisphosphate $[M-2H]^{2-}$ ion confirm the fragmentation pattern of inositol phosphates in the ESI. Further evidence for dissociation in the ion source is shown by the effect of increasing the source voltage on the mass spectrum of phytic acid. The ESI-HRMS analysis of inositol phosphates is unusual and highly characteristic. The study of the full mass spectrum of IP6 in ESI-HRMS mode indicates the detection of the compound in environmental matrices using this technique is preferable to multiple reaction monitoring (MRM).

3.2 Introduction

Frequently reported as the most abundant organic P compound found in soils and sediments, IP6 is one of the more important organic P compounds in the environment and as such was chosen as a model compound for the development and testing of methodology for the characterisation of organic P using ESI-HRMS. The compound is unusual in its structure (Figure 3.1 A), containing a constrained six-membered carbon ring, high electron density phosphate groups, and having the potential to form up to twelve negative charges, depending on the pH of solution. It was decided to first characterise the standard IP6 compound using ESI-HRMS and investigate its behaviour under ESI conditions.

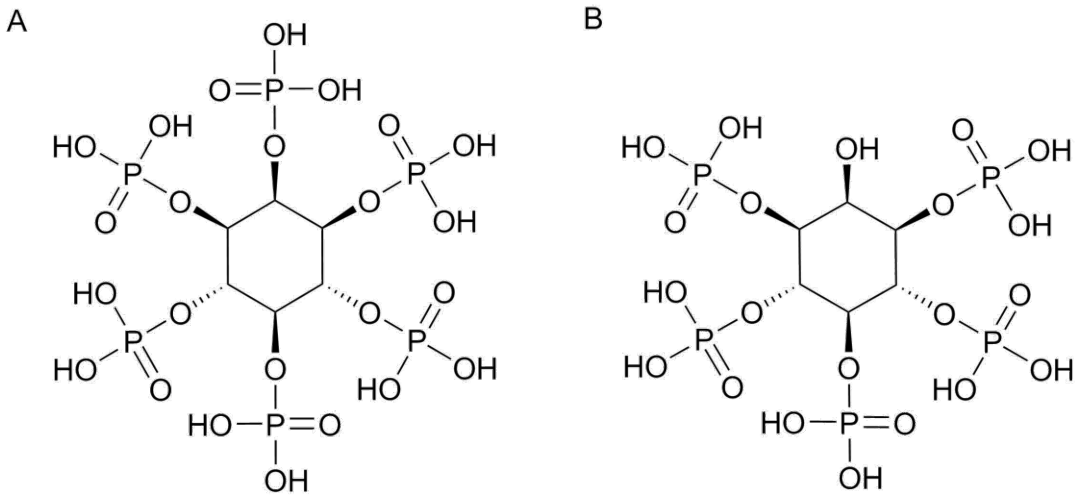
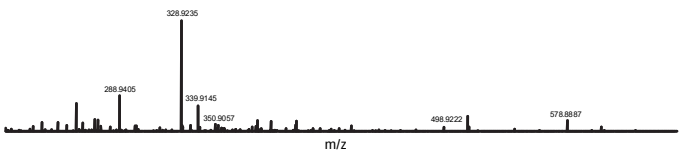


Figure 3.1. (A) The structures of *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP6) and (B) *myo*-inositol 1,3,4,5,6-pentakisphosphate (IP5) as determined by Johnson & Tate (1969). Monoisotopic masses are 659.8614 Da and 579.8950 Da, respectively.

Due to its importance, IP6 has been widely studied. It was first extracted in 1895 (Plimmer & Page, 1913), with studies in the following decades attempting to determine its chemical formula (Anderson, 1920). Despite the structure of phytic acid not being confirmed until 1969 by ^{31}P NMR (Johnson & Tate, 1969) and 1971 by X-ray crystallography (Blank & Sax, 1971), it has been extensively studied and shown to be present in high concentrations in grains, seeds and



plant roots (Van Steveninck *et al.*, 1987). Due to its high abundance in soils, IP6 potentially plays an important role in the P biogeochemical cycle and indeed in the P enrichment of water bodies *via* transport from soils and sediments.

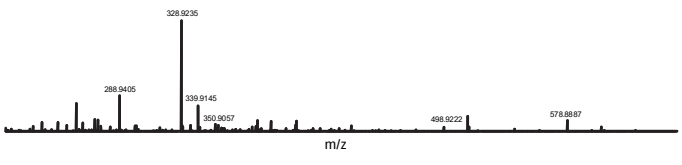
Early determinations of IP6 were made *via* post acid extraction from seeds, followed by precipitation of IP6 with Fe(III) and measurement of the depleted concentration of Fe(III) in solution, thereby inferring the concentration of IP6 (Ellis *et al.*, 1977). In 1977 Harland and Oberleas (1977) demonstrated the hydrolysis of IP6 from soybeans using concentrated H_2SO_4 and HNO_3 and the quantification of the released phosphate using the molybdenum blue test (Murphy & Riley, 1962). The determination of IP6 along with the inositol phosphate stereoisomers from soils by Cosgrove (1962) in the 1960s was achieved by the hydrolysis of the inositol phosphates followed by paper chromatography of the inositol core. An alternative method for the determination of IP6 using phytase enzymatic digestion has also been used widely (He *et al.,* 2006); the concentration of phosphate released from the digested IP6 is measured using molybdenum colorimetry. Phytases may however, not be IP6-specific, and may digest other phosphate-containing compounds co-occurring in complex environmental matrices.

In recent decades, a range of more specific analytical methods for determining IP6 have been developed. Liquid or anion exchange chromatography has been used to separate, identify and quantify inositol phosphates in food and biological samples on the basis of retention time and peaks areas (Talamond *et al.*, 2000; Shelor *et al*., 2015). The methods have contended with the presence of the homologous compounds, the lower inositol phosphates (IPxs) e.g. pentakisphosphate, tetrakisphosphate etc., and the stereoisomers of the inositol phosphates in the chiro, scillo, neo, etc. forms making chromatographic separation of the compounds difficult. These lower inositol phosphates are intermediates in the biosynthesis of IP6, and so

are commonly found associated with IP6 in plant extracts. In ion exchange chromatography systems IP6 detection uses electrochemical conductivity detection (Waithasong *et al.*, (2015), or post-column derivitisation with $Fe(NO_3)_3$ for spectrophotometric detection (Chen & Li, 2003). Liquid chromatographic systems have also used refractive index detection of IP6 (Tangendjaja *et al.*, 1980), or more recently inductively-coupled plasma mass spectrometry (Persson *et al*., 2009). Surprisingly few studies (see below) have employed direct determination of IP6 using mass spectrometry, perhaps due to the fact that ion exchange chromatography liquid chromatography (LC) systems are generally incompatible with mass spectrometers due to metal components in the interface pumping systems and the high ionic strength of mobile phases.

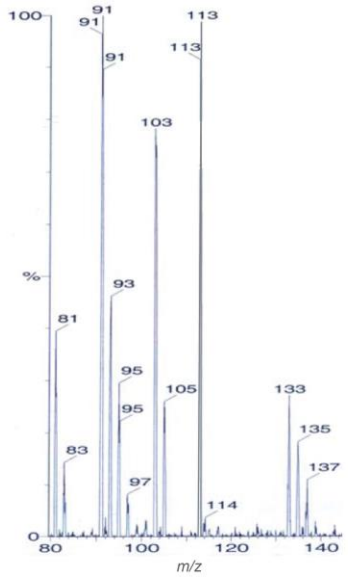
Currently, ^{31}P NMR spectroscopy is the principal method of characterisation of P in matrices such as soils and manures (Cade-Menun & Liu, 2014, Turner *et al.*, 2003c, Turner, 2004). This method is, however, compromised by the low sensitivity of NMR. Furthermore, the complexity of soil extracts results in multiple overlapping resonances in the diagnostic regions of the NMR spectrum. This makes unequivocal identification of individual compounds difficult (Doolette & Smernik, 2015), particularly if their relative concentrations are low. Identifications by ^{31}P NMR spectrum in soil extracts usually rests on comparisons of resonances with literature values (Turner 2004), or spiking experiments (Cade-Menun *et al.*, 2010). In the absence of knowledge of the numbers and abundances of compounds contributing to an NMR spectrum, it is conceivable that the peaks identified as correlating to IP6 may derive from a number of unknown compounds which happen to have similar chemical shifts to IP6. The specificity of this method is therefore open to debate.

Few studies appear in the literature regarding the use of ESI-MS for the analysis of inositol phosphates in environmental extracts. One aspect of ESI is the formation of salt adducts with



ions present in the electrospray solution. These adducts can result in multiple analyte-adduct ions, complicating the interpretation of the recorded spectra and reducing ion yields. This is particularly relevant in the case of IP6 where there is potential for the compound to form adducts with up to twelve cations. The complexity this adds to the identification of IP6 using ESI-MS is evident in Heighton *et al*. (2008), whereby cations were added to the IP6 solution in order to use the formation of adducts to identify acid dissociation constants. Up to 16 ions are identified as IP6 per cluster (Figure 3.2 A) in the spectrum with Fe^{3+}, Na^{+} or Cu^{2+} adducts, or a mixture of these metals. Figure 3.2 (B) illustrates an example of the ions identified after addition of cupric chloride. The addition of these different metals complicated, rather than aided, the interpretation of the mass spectra.

A



B

pH 6				
Peak assignment	*z*	*m/z*	*m*	Charge
$C_6H_6Cu^{63}{}_2Na_2O_{24}P_6$	-6	137	820	-12+4 +2,
$C_6H_8Cu^{63}{}_2O_{26}P_6$	-6	135	808	-12+4+2
$C_6H_7Cu^{63}{}_2NaO_{24}P_6$	-6	133	798	-12+4+1+1
$C_6H_{11}NaO_{24}P_6$	-6	113	676	-12+1+5
$C_6H_{10}Na_7O_{19}P_6$	-7	105	733	-12+7+5
$C_6H_{12}Na_5O_{21}P_6$	-7	103	721	-12+5+7
$C_6H_6Cu^{63}Na_2O_{25}P_6$	-8	97	773	-12+2+2
$C_6H_6Cu^{63}Na_2O_{24}P_6$	-8	95	757	-12+2+2
$C_6H_8Cu^{63}O_{26}P_6$	-8	93	745	-12+2+2
$C_6H_8Cu^{63}O_{25}P_6$	-8	91	729	-12+2+2
$C_6H_{10}O_{25}P_6$	-8	83	668	-12+4
$C_6H_{10}O_{24}P_6$	-8	81	652	-12+4

Figure 3.2. (A) ESI-MS negative ion mass spectrum of cupric chloride and IP6 at pH 6 in formic acid mobile phase. (B) peak assignments for ions identified in A. Adapted from Heighton *et al.,* 2008.

Rougemont *et al.* (2016) developed a method whereby ion pair chromatography was used to separate IP6 extracted from a whole blood matrix. The addition of pentylamine and hexafluoroisopropanol modifiers to the LC eluent resulted in fewer adducts, and lower

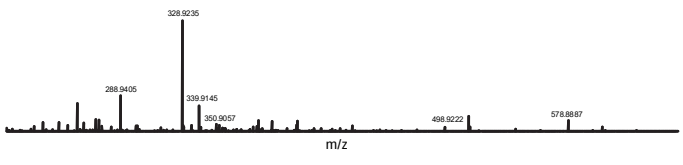
fragmentation and therefore simplified the mass spectra and improved the identification of IP6. Accurate mass analysis was, however, not employed in this study, nor was the behaviour of IP6 under ESI conditions studied.

The information gathered from the analysis in this chapter highlight a difficulty encountered in studies of inositol phosphates whereby LC-MS with multiple reaction monitoring (MRM) is used to detect individual species. If inositol phosphates fragment under the ESI conditions to produce ions that are isobaric with lower inositol phosphates, then the use of MRM detection will produce false positive results as fragments are misidentified as lower IPxs. In two studies, Paraskova *et al.* (2015) and Sjoberg *et al.* (2016), MRM was used to detect IPx compounds in sediments on the basis of a set of transitions for each species. In-source fragmentation resulted in overlapping IPx peaks in the chromatogram and matrix effects reduced the chromatographic resolution. These effects complicate the identification and quantification of IPxs. Where isomers of IPx elute at unpredicted retention times, their fragmentation may interfere with detection of lower IPxs.

3.3 Aims

The aims of this chapter were:

1. Study the negative ion ESI-HRMS of IP6 using Orbitrap ESI-HRMS and determine the mass spectrum of the standard and investigate the impact of impurities in the mass spectrum.
2. Explore the MS/MS fragmentation patterns of the characteristic ions.
3. Rationalise the behaviour of IP6 in the electrospray source by comparison to IP5 reference standard (Figure 3.1 B) and the MS/MS spectra and by changing of the source conditions.



4. To use preparatory IC to overcome the complications caused by the impurity of the IP6 reference standard.

The unusual behaviour of the compound in the electrospray source of the Orbitrap was thus identified and described.

3.4 Methods

3.4.1 Standard compounds

Reference standards IP6 (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate sodium salt, $Na_{12}C_6H_{12}O_6(HPO_3)_6$) and IP5 (*myo*-inositol 1,3,4,5,6-pentakisphosphate pentapotassium salt, $K_5C_6H_{12}O_6(HPO_3)_5$) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Solutions (20 ppm for MS, 10 ppm for qualitative IC, 130 ppm for preparative IC) were prepared with double distilled water.

3.4.2 Ion chromatography

A Dionex ICS-5000 (Thermo Scientific, Hemel Hempstead, UK) ion chromatograph equipped with KOH eluent generator, ion suppressor and conductivity detector was used for chromatographic separation, identification and quantification of reference standards IP6 and IP5. Compounds were separated using an Ionpac AS11 (2 mm x 250 mm) column (Thermo Scientific, Hemel Hempstead, UK), with AS11G guard column (2 mm x 50 mm). The flow rate was set to 0.250 mL min^{-1}, and column temperature to 30 °C. The elution gradient comprised an initial 10 min equilibration at 4 mM KOH, followed by 0 to 19 min: 4 mM KOH, 19 to 24 min: ramp to 70 mM, 24 to 29 min: 70 mM KOH, 29 to 30 mM: ramp 4 mM KOH. Eluate fractions were collected post-detection at 30 s intervals in glass vials for HRMS analysis. Chromatograms were analysed in Chromeleon (Thermo Scientific, Hemel Hempstead, UK).

3.4.3 High resolution accurate mass spectrometry

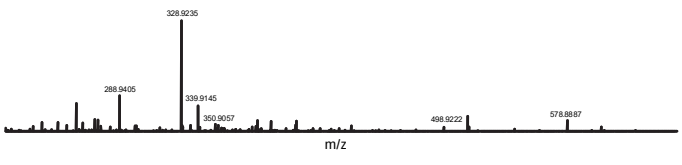
MS analysis of IP6 and IP5 was performed on an Orbitrap Elite™ (Thermo Scientific, Hemel Hempstead, UK) with ESI source. The Orbitrap was operated in negative ion mode, calibrated using negative ion calibration solution (Thermo Scientific, Hemel Hempstead, UK) and tuned automatically on the *m/z* 328.9 (IP6 $[M–2H]^{2–}$ ion). Solutions were directly infused at 10 $\mu l.min^{-1}$ for acquisition of initial full mass spectra and at 6 $\mu L\ min^{-1}$ for MS/MS analysis. The source voltage was set to −1.8 kV, sheath gas (nitrogen) flow rate to 30 arbitrary units (arb), auxiliary gas (nitrogen) to 0 arb and sweep gas (nitrogen) to 1 arb. The capillary temperature was 275 °C. Full mass spectra were recorded at 120,000 resolution and 50 scans were averaged in order to increase the signal-to-noise ratio. MS/MS spectra were recorded at 15,000 resolution for a higher scan rate. MS/MS data was collected for the top 20 ions in a spectrum. Fragmentation was *via* higher energy collisional dissociation (HCD) at 65 % normalised energy. Mass spectra were analysed using Xcalibur version 3.0.63 (Thermo Scientific, Hemel Hempstead, UK).

The effect of varying the source voltage was studied by maintaining sheath gas at 30 arb, auxiliary at 0 arb, and sweep gas at 1 arb, and changing the source voltage from −1.0 kV to −3.6 kV in 0.2 kV increments.

IC fraction solutions were directly infused at 10 $\mu l.min^{-1}$. The source voltage was set to −3.4 kV, sheath gas flow rate to 30 arb, auxiliary gas to 15 arb and sweep gas to 9 arb.

3.5 Results and Discussion

P-containing compounds such as phosphorylated proteins and phospholipids are commonly studied in biological systems using ESI-MS. For example, in the characterisation of phospholipids the polar head group and fatty acyl substituents may be determined and used in the study of microbial ecology (Sturt *et al.*, 2004). Identification of phosphorylation sites on



peptides by the loss of phosphate in MS/MS informs the study of post translational modifications (Eyrich *et al.,* 2011). Often targeted studies are those that seek to identify and quantify anthropogenic P compounds such as flame retardants in wastewater (Rodil *et al.,* 2005). There is very little discussion in the literature of the MS of naturally occurring environmental P compounds. Choi *et al.* (2000) characterised polyphosphates, which occur in wastewater effluent and agricultural run-off, using negative ion ESI-MS and noted they fragment at higher potential settings. However, this study is the first in-depth characterisation of the behaviour of an important organic soil P compound using ESI-HRMS.

As discussed in the previous chapter, the phosphate moiety of organic P compounds imparts anionic properties to the compounds and are thus more suited to negative ion MS. This is especially true for the inositol phosphates which contain multiple phosphate groups and, in the case of IP6, may bear 12 negative charges depending on the pH of the solution.

3.5.1 ESI-HRMS mass spectrum of IP6

The full negative ion ESI-HRMS mass spectrum of IP6 is given in Figure 3.3 (A) and a list of the major ions identified in the mass spectrum are given in Table 3.1. The ions demonstrate the propensity of IP6 for charge acquisition with doubly- (a, b, c, d, e, f, g, h) and singly-charged (i, j, k, l, m, n, o), species occurring in the mass spectrum. The major intact IP6 ion observed is the doubly-charged ion, $[M-2H]^{2-}$ (e, *m/z* 328.9217), with no singly charged $[M-H]^{-}$ ion appearing in the spectrum (theoretical *m/z* 658.8535) as would ordinarily be expected using ESI. There is indication that IP6 readily accumulates sodium ions forming adducts of both singly- and doubly-charged ions (b, d, f, g, h, j, m, n). The concentration of electronegative phosphate moieties on the compound makes these unsurprising occurrences.

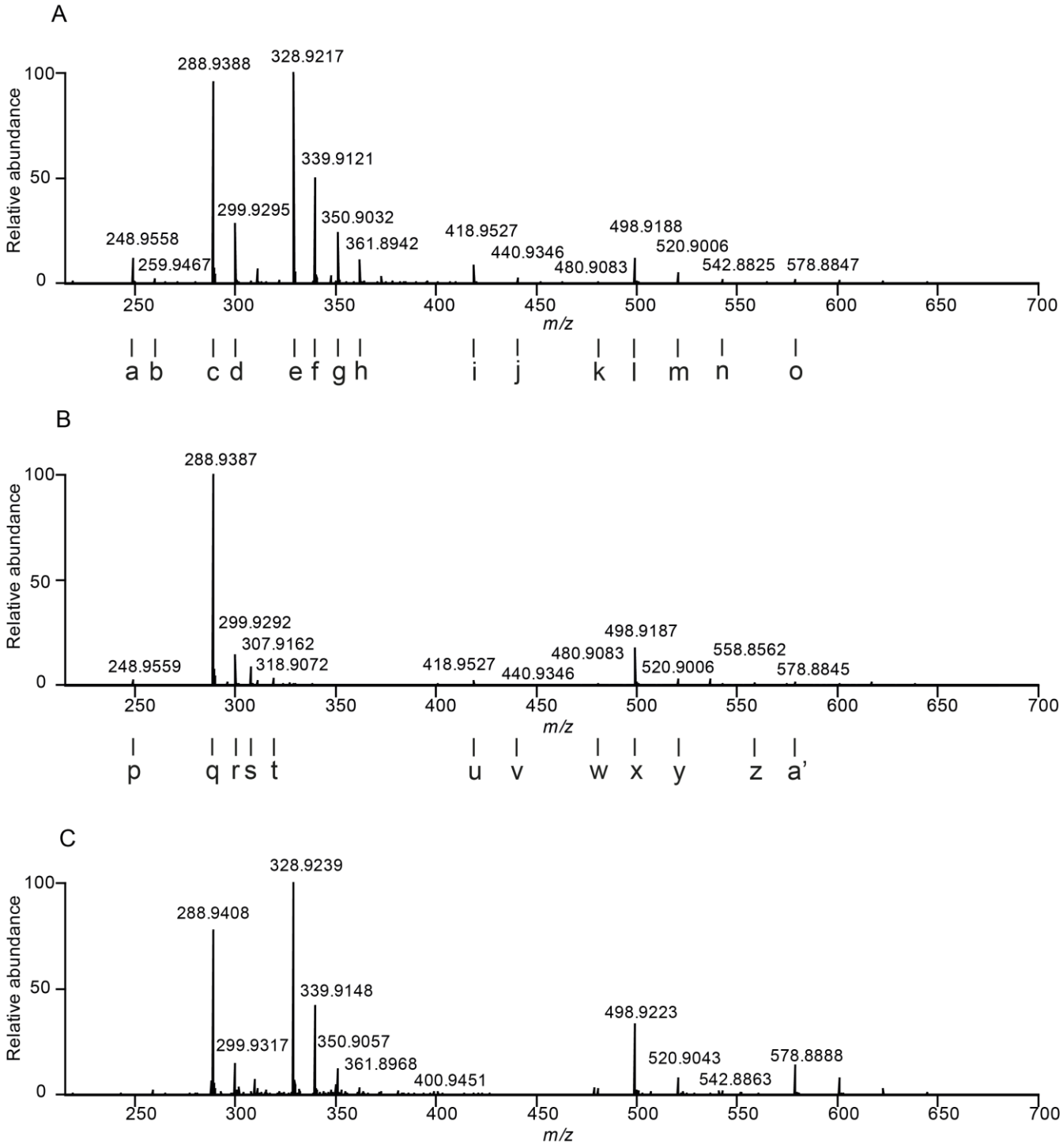


Figure 3.3. Negative ion mass spectra obtained by direct infusion on an ESI-Orbitrap HRMS: (A) IP6 reference standard, (B) IP5 reference standard, and (C) isolated IP6 in fraction 1 (F1, Figure 3.5 B). Ions a to a' are detailed in Table 3.1.

Table 3.1. Ions, charge, formula and mass accuracy (ppm) in the full mass spectra of IP6 and IP5 (Figure 3.3 A and 3.3 B respectively). The RHS column indicates the precursor ions giving rise to the fragment ions deduced by MS/MS.

	Ion	z	Formula	ppm	Precursor ion (MS/MS)
	IP6 Spectrum (Fig 3.3-A)				
a†	$[M-2HPO_3-2H]^{2-}$	2	$C_6H_{14}O_{18}P_4$	1.5	
b	$[M-2HPO_3-3H+Na]^{2-}$	2	$C_6H_{13}O_{18}P_4Na$	1.5	
c	$[M-HPO_3-2H]^{2-}$	2	$C_6H_{15}O_{21}P_5$	1.5	
d	$[M-HPO_3-3H + Na]^{2-}$	2	$C_6H_{14}O_{21}P_5Na$	1.9	
e	$[M-2H]^{2-}$	2	$C_6H_{16}O_{24}P_6$	1.8	
f	$[M-3H+Na]^{2-}$	2	$C_6H_{15}O_{24}P_6Na$	2.5	
g	$[M-4H+2Na]^{2-}$	2	$C_6H_{14}O_{24}P_6Na_2$	2.3	
h	$[M-5H+3Na]^{2-}$	2	$C_6H_{13}O_{24}P_6Na_3$	2.2	
i	$[M-3HPO_3-H]^{-}$	1	$C_6H_{14}O_{15}P_3$	4.4	248.96, 288.94, 328.92,498.92
j	$[M-3HPO_3-2H+Na]^{-}$	1	$C_6H_{13}O_{15}P_3Na$	4.3	299.93
k	$[M-2HPO_3-H_2O-H]^{-}$	1	$C_6H_{13}O_{17}P_4$	4.2	288.94
l	$[M-2HPO_3-H]^{-}$	1	$C_6H_{15}O_{18}P_4$	4.2	288.94, 328.92
m	$[M-2HPO_3-2H+Na]^{-}$	1	$C_6H_{14}O_{18}P_4Na$	4.3	299.93
n	$[M-2HPO_3-3H+2Na]^{-}$	1	$C_6H_{13}O_{18}P_4Na_2$	4.2	
o	$[M-HPO_3-H]^{-}$	1	$C_6H_{16}O_{21}P_5$	4.3	328.92
	IP5 Spectrum (Fig 3.3 B)				
p	$[M-HPO_3-2H]^{2-}$	2	$C_6H_{14}O_{18}P_4$	1.3	
q	$[M-2H]^{2-}$	2	$C_6H_{15}O_{21}P_5$	1.7	
r	$[M-3H+Na]^{2-}$	2	$C_6H_{14}O_{21}P_5Na$	2.4	
s	$[M-3H+K]^{2-}$	2	$C_6H_{14}O_{21}P_5K$	2.3	
t	$[M-4H+NaK]^{2-}$	2	$C_6H_{13}O_{21}P_5K$	2.2	
u	$[M-2HPO_3-H]^{-}$	1	$C_6H_{14}O_{15}P_3$	4.4	
v	$[M-2HPO_3-2H+Na]^{-}$	1	$C_6H_{13}O_{15}P_3Na$	4.3	248.96, 288.94, 498.92
w	$[M-HPO_3-H_2O-H]^{-}$	1	$C_6H_{13}O_{17}P_4$	4.2	
x	$[M-HPO_3-H]^{-}$	1	$C_6H_{15}O_{18}P_4$	4.4	288.94
y	$[M-HPO_3-2H+Na]^{-}$	1	$C_6H_{14}O_{18}P_4Na$	4.3	288.94
z	$[M-HPO_3-2H+Na+K]^{-}$	1	$C_6H_{13}O_{18}P_4NaK$	4.5	299.93
a’	$[M-H]^{-}$	1	$C_6H_{15}O_{21}P_5$	4.7	

†Letters correspond to annotated ions in the mass spectra shown in Figure 3.3.

Ions which appear to be fragments of the IP6 molecular ion corresponding to the chemical formula $[IP6 - xHPO_3 - yH]^{y-}$ (a, c, i, l, o, Figure 3.3 A) are isobaric with lower inositol phosphate (IP5, IP4, IP3) ions. Therefore, lower inositol phosphate impurities in the reference standard may account for the presence of these ions in the mass spectra. The ion chromatogram (Figure 3.4 A) of the reference standard indicates that the IP6 standard is not pure.

Alternatively, the lower inositol phosphate ions may be formed under ESI through the loss of phosphate due to in-source fragmentation of the IP6 ion, possibly *via* a 1,3-hydride shift previously reported by Palumbo *et al.* (2011). The observation of ions corresponding to the loss of water and phosphate (k, w, Figure 3.3 A) also potentially indicate in-source fragmentation.

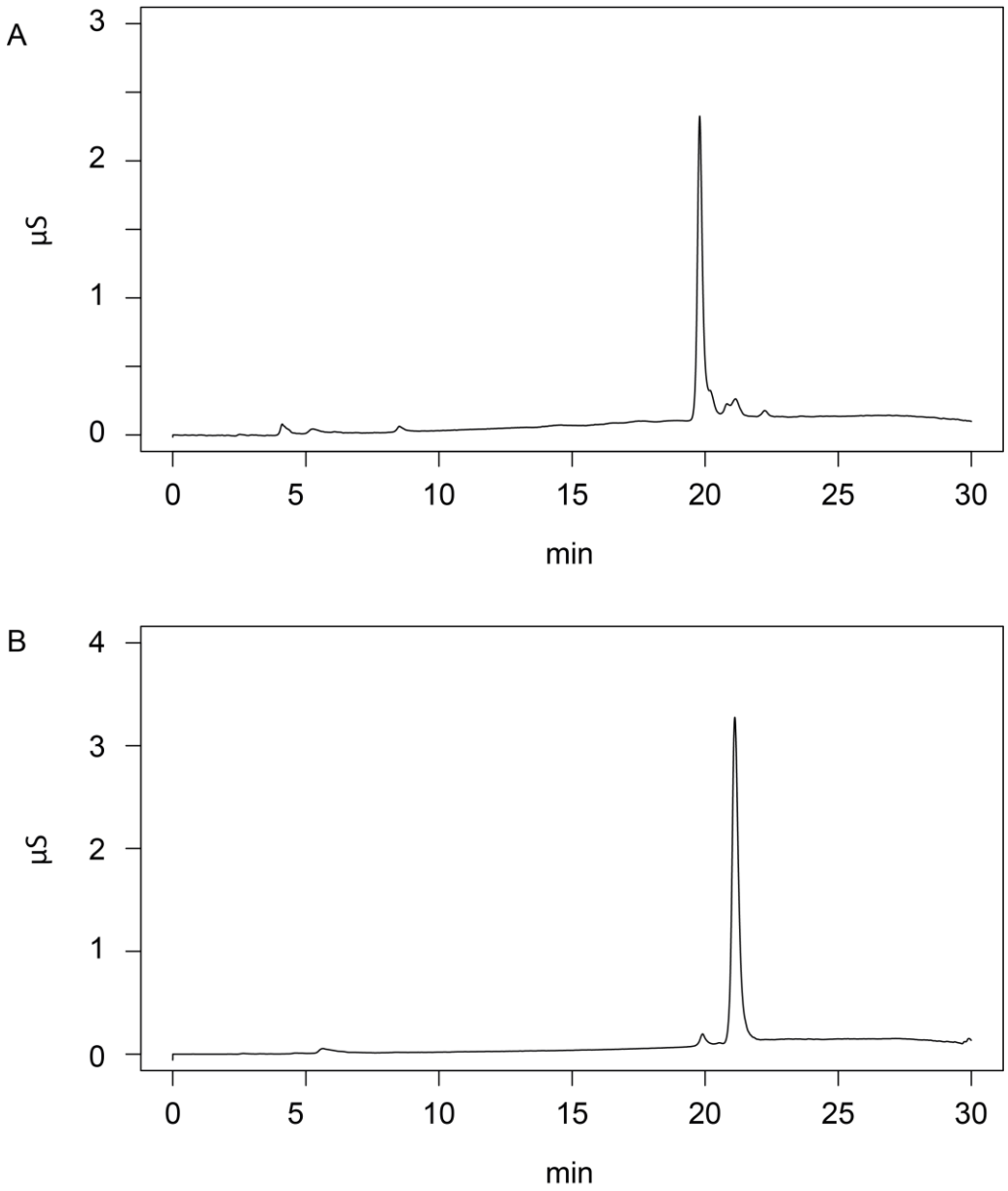
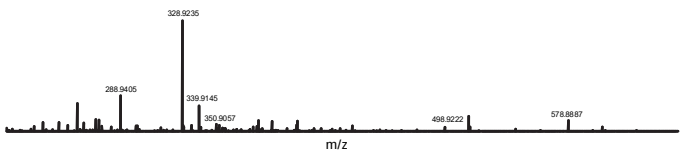


Figure 3.4. Ion chromatograms of (A) IP6 and (B) IP5 reference standards (10 ppm) obtained on a Dionex ICS‐5000 with an Ionpac AG11 column and KOH eluent. The IP6 standard is ~84% pure, while the IP5 standard is ~97% pure with IP6 contamination at ~20 min.



This configuration of ions in the negative ion HRMS mass spectrum of IP6 reveals charge acquisition, fragmentation and adduct-forming behaviour under ESI conditions. The implications of this behaviour are particularly relevant where the use of MRM or selected ion mass chromatography is used for identification of inositol phosphates.

We therefore sought to confirm or refute the in-source fragmentation by purifying the reference standard and comparison of the IP6 mass spectrum with that of an IP5 reference standard which was shown to be ~ 97 % pure (Figure 3.4 B). The fragmentation behaviour of the IP6 and IP5 $[M-2H]^{2-}$ ions was explored using tandem MS.

3.5.2 ESI-HRMS mass spectrum of IP5

The HRMS negative ion mass spectrum of the IP5 reference standard is given in Figure 3.3 B. The major ions (Table 3.2) include the doubly-charged ion, $[M-2H]^{2-}$ (q, m/z = 288.9387), and sodium and potassium adducts of these ions (r, s, t). The IP5 standard was in the form of a potassium salt ($K_5C_6H_{12}O_6(HPO_3)_5$) which accounts for these adducts. Such is the propensity of inositol phosphates to form adducts, that the compound readily combines with the ambient Na ions in solution.

3.5.3 IP6 purification with IC

Purification of the IP6 reference compound was achieved by collecting eluate fractions from the ion chromatograph corresponding to 30 second intervals in the ion chromatogram (Figure 3.5). The mass spectrum of the leading front edge of the IP6 peak (the purest IP6 fraction F1, Figure 3.5 B) is given in Figure 3.3 C and is very similar to that of the IP6 reference standard, confirming that the IP5 and IP4 observed in the reference spectra most likely arise from in-source fragmentation of IP6 and not from contamination.

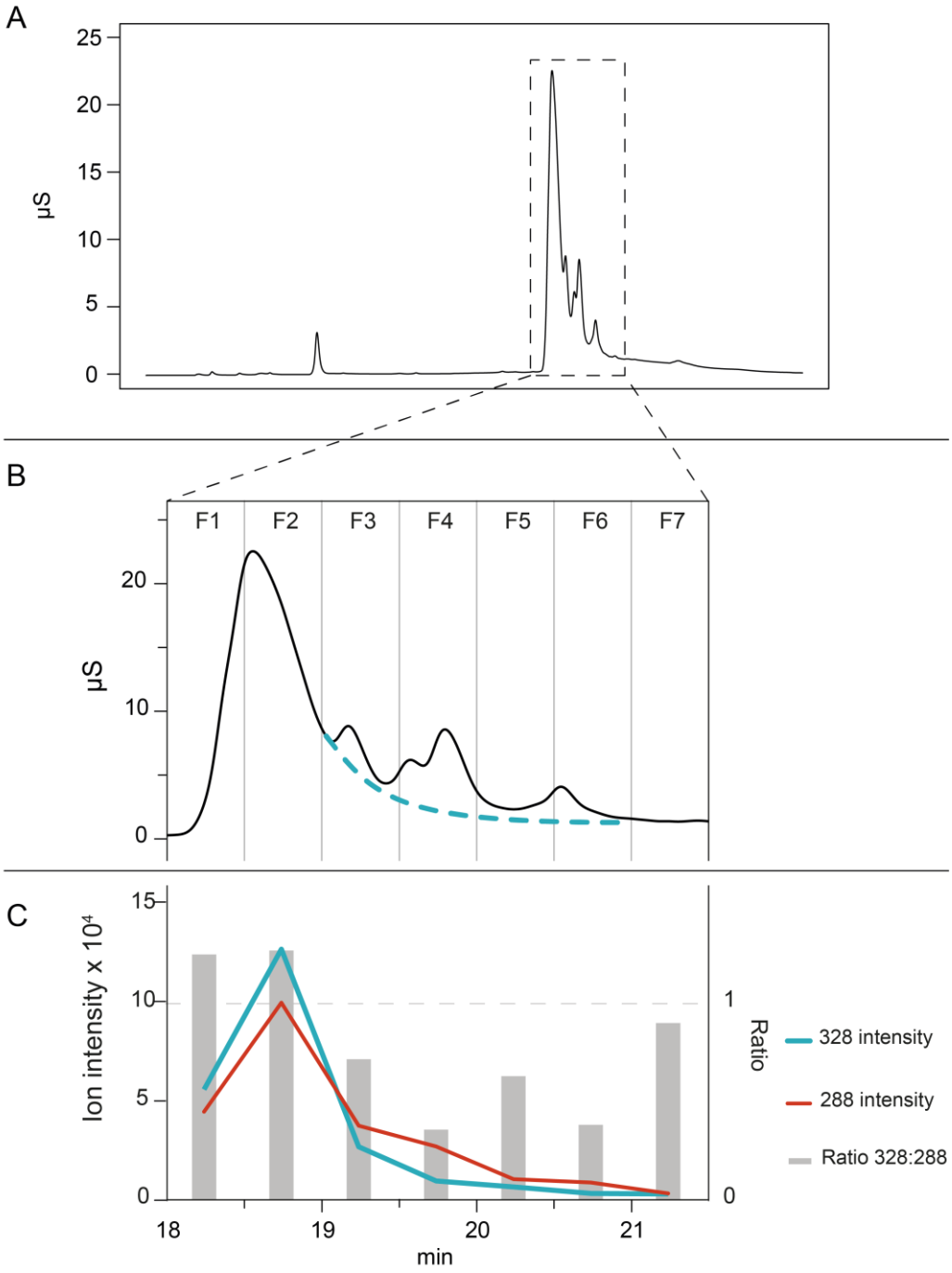
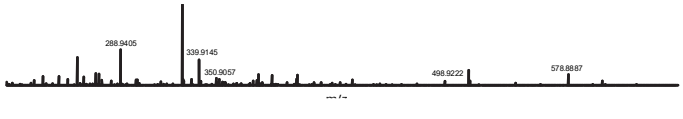


Figure 3.5. (A) full ion chromatogram of 130 ppm IP6 reference standard, with enlarged region 18 to 21.5 min and fractions delineated in (B). Fractions were collected from the IC system in 30 s intervals. Mass spectra of each fraction were then obtained by direct infusion to the Orbitrap HRMS in negative ion mode. (C) corresponding ion intensities for *m/z* 328.92 (IP6) and *m/z* 288.94 (IP5 or IP6 $[M-HPO_3-2H]^{2-}$) in each fraction, along with the ratio of those ions. The IP6 peak is extrapolated (dotted line) in (B) on the basis of the presence of the *m/z* 328.92 ion in the mass spectra.



The mass spectra of the eluate corresponding to the following 30 second fractions suggest that the IP6 peak tails into the later eluting peaks as depicted in Figure 3.5 B. The basis for this extrapolation is that IP6 appears in each of the fractions, although its abundance diminishes from Fraction 3 to Fraction 7. In these fractions the major ion is IP5 $[M-2H]^{2-}$. The ratio of the ions *m/z* 328:288, i.e. IP6 $[M-2H]^{2-}$: IP5 $[M-2H]^{2-}$, was determined for each of the fractions and this is illustrated in Figure 3.5 C. The ratio is constant for Fraction 1 and 2 as the IP6 peak elutes. In the following fractions, the ratio of *m/z* 328:288 falls below 1, as IP5 $[M-2H]^{2-}$ comes to dominate the mass spectra. This shows that the minor peaks in the chromatogram are isomers of IP5, and that the IP6 elutes from the column in Fractions 1 and 2, then tails through the chromatogram to Fraction 7. The larger peak in Fraction 4 was determined to be the IP5 reference standard, confirmed by co-injection of the IP6 and IP5 reference standards. On this basis, the purity of the IP6 reference standard was calculated to be ~ 84 % with lower inositol phosphates forming the majority of the impurities, along with a small amount of phosphate (~ 7 min).

3.5.4 MS/MS fragmentation

For further confirmation of the source of the fragment ions in the full mass spectrum, the fragmentation pattern of the IP6 and IP5 $[M-2H]^{2-}$ ions were studied *via* tandem MS experiments under HCD conditions. The MS/MS spectra are shown in Figure 3.6, with identified ions detailed in Table 3.2. The MS/MS spectrum of the *m/z* 288.94 ion from the IP6 reference standard is also presented for comparison. Analysis of the MS/MS fragmentation patterns of the precursor ions *m/z* 328.92 and *m/z* 288.94 demonstrate that the principal form of fragmentation is the loss of HPO_3. Loss of water from the precursor ion is more prevalent in these MS/MS spectra than in the full MS, indicative of the higher energy conditions in the HCD cell, compared to those in the ESI source. Precursor ions that give rise to product ions identified

as fragments in the full MS are indicated in Table 3.1. Many of the ions have more than one precursor illustrating the need for careful consideration in the assertion of the source of fragment ions.

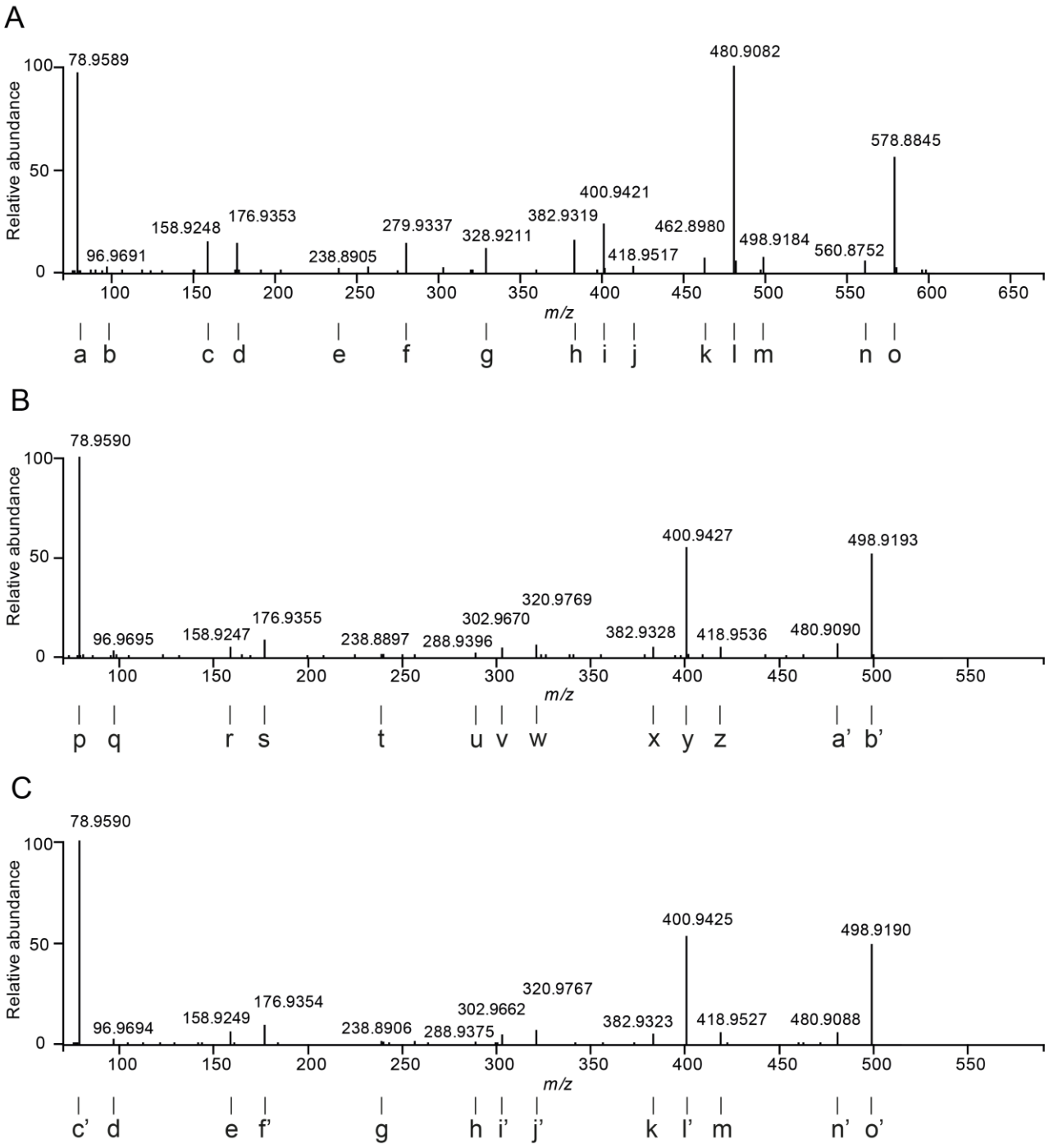


Figure 3.6. Fragmentation MS/MS HCD 65% NCE scans of precursor ions: (A) *m/z* 328.92 (IP6 standard), (B) *m/z* 288.94 (IP6 standard), and (C) *m/z* 288.94 (IP5 standard). Ions a‑o' are detailed in Table 3.2.

Table 3.2. Ions, charge, formula and mass accuracy (ppm) in the MS/MS product spectra of *m/z* 328.92 (IP6 standard), 288.94 (IP6 standard), and 288.94 (IP5 standard) (Figure 3.6 A, B and C, respectively).

	Ion	z	Formula	ppm
	328.94 product ion spectrum			
a†	Phosphate	1	PO_3	−10.32
b	Phosphate	1	H_2PO_4	−8.34
c	Polyphosphate	1	HP_2O_6	−5.0
d	Polyphosphate	1	$H_3P_2O_7$	−0.3
e	Polyphosphate	1	$H_2P_3O_9$	0.2
f	$[M-HPO_3-H_2O-2H]^{2-}$	2	$C_6H_{13}O_{20}P_5$	0.6
g	$[M-2H]^{2-}$	2	$C_6H_{16}O_{24}P_6$	2.8
h	$[M-3HPO_3-2H_2O-H]^-$	1	$C_6H_{10}O_{13}P_3$	1.3
i	$[M-3HPO_3-H_2O-H]^-$	1	$C_6H_{12}O_{14}P_3$	2.7
j	$[M-3HPO_3-H]^-$	1	$C_6H_{14}O_{15}P_3$	4.0
k	$[M-2HPO_3-2H_2O-H]^-$	1	$C_6H_{11}O_{16}P_4$	4.7
l	$[M-2HPO_3-H_2O-H]^-$	1	$C_6H_{13}O_{17}P_4$	6.8
m	$[M-2HPO_3-H]^-$	1	$C_6H_{15}O_{18}P_4$	0.8
n	$[M-HPO_3-H_2O-H]^-$	1	$C_6H_{14}O_{20}P_5$	4.4
o	$[M-HPO_3-H]^-$	1	$C_6H_{16}O_{21}P_5$	5.0
	288.94 IP6 product ion spectrum			
p	Phosphate	1	PO_3	−6.3
q	Phosphate	1	H_2PO_4	−4.4
r	Polyphosphate	1	HP_2O_6	0.9
s	Polyphosphate	1	$H_3P_2O_7$	−0.6
t	Polyphosphate	1	$H_2P_3O_9$	6.1
u	$[M-2H]^{2-}$	2	$C_6H_{15}O_{21}P_5$	0.2
v	$[M-3HPO_3-2H_2O-H]^-$	1	$C_6H_9O_{10}P_2$	0.3
w	$[M-3HPO_3-H_2O-H]^-$	1	$C_6H_{11}O_{11}P_2$	2.4
x	$[M-2HPO_3-2H_2O-H]^-$	1	$C_6H_{10}O_{11}P_3$	1.6
y	$[M-2HPO_3-H_2O-H]^-$	1	$C_6H_{12}O_{14}P_3$	3.2
z	$[M-2HPO_3-H]^-$	1	$C_6H_{14}O_{15}P_3$	2.3
a'	$[M-HPO_3-H_2O-H]^-$	1	$C_6H_{13}O_{17}P_4$	2.7
b'	$[M-HPO_3-H]^-$	1	$C_6H_{15}O_{18}P_4$	3.2
	288.94 IP5 product ion spectrum			
c'	Phosphate	1	PO_3	−6.3
d'	Phosphate	1	H_2PO_4	−3.4
e'	Polyphosphate	1	HP_2O_6	−0.4
f'	Polyphosphate	1	$H_3P_2O_7$	0.0
g'	Polyphosphate	1	$H_2P_3O_9$	2.4
h'	$[M-2H]^{2-}$	2	$C_6H_{15}O_{21}P_5$	3.8
i'	$[M-3HPO_3-2H_2O-H]^-$	1	$C_6H_9O_{10}P_2$	3.0
j'	$[M-3HPO_3-H_2O-H]^-$	1	$C_6H_{11}O_{11}P_2$	3.0
k'	$[M-2HPO_3-2H_2O-H]^-$	1	$C_6H_{10}O_{11}P_3$	2.9
l'	$[M-2HPO_3-H_2O-H]^-$	1	$C_6H_{12}O_{14}P_3$	3.7

m'	$[M-2HPO_3-H]^-$	1	$C_6H_{14}O_{15}P_3$	4.4
n'	$[M-HPO_3-H_2O-H]^-$	1	$C_6H_{13}O_{17}P_4$	3.2
o'	$[M-HPO_3-H]^-$	1	$C_6H_{15}O_{18}P_4$	3.8

† Letters correspond to annotated ions in the mass spectra shown in Figure 3.6.

3.5.5 Source Voltage Effects on Fragmentation

The effect of changing the source voltage on the pattern of ions observed was investigated and the results are presented in Figure 3.7. Varying the voltage from 1.0 – 1.4 kV, revealed the stability of the spray was poor with low ion intensity. The voltage was not raised above 3.6 kV as arc discharge in the source was observed at these voltages. Varying the voltage between 1.6 and 3.6 kV shows a clear trend emerge whereby the relative abundance of the doubly charged IP6 $[M-2H]^{2-}$ ion decreases, while the relative abundances of the *m/z* 288.94 and *m/z* 248.96 increase, with *m/z* 288.94 becoming the dominant ion in the spectra. This reinforces the idea that *m/z* 288.94 and *m/z* 248.96 are fragments of the IP6 $[M-2H]^{2-}$ ion and that increasing the voltage in the electrospray source increases the extent of fragmentation of the compound.

Taken together: the mass spectra of the two inositol phosphate reference standards, the MS data from the ion chromatographically purified compound, the MS/MS data, and the effect of the source voltage on the mass spectrum of IP6 – all demonstrate that the compound acquires charge and readily fragments in-source under ESI conditions with the loss of HPO_3 and water. The fragment ions are isobaric with lower inositol phosphates and could therefore be mistaken for presence of these compounds in a sample. Knowledge of the behaviour of inositol phosphates in ESI-HRMS is essential for informed analysis of these compounds.

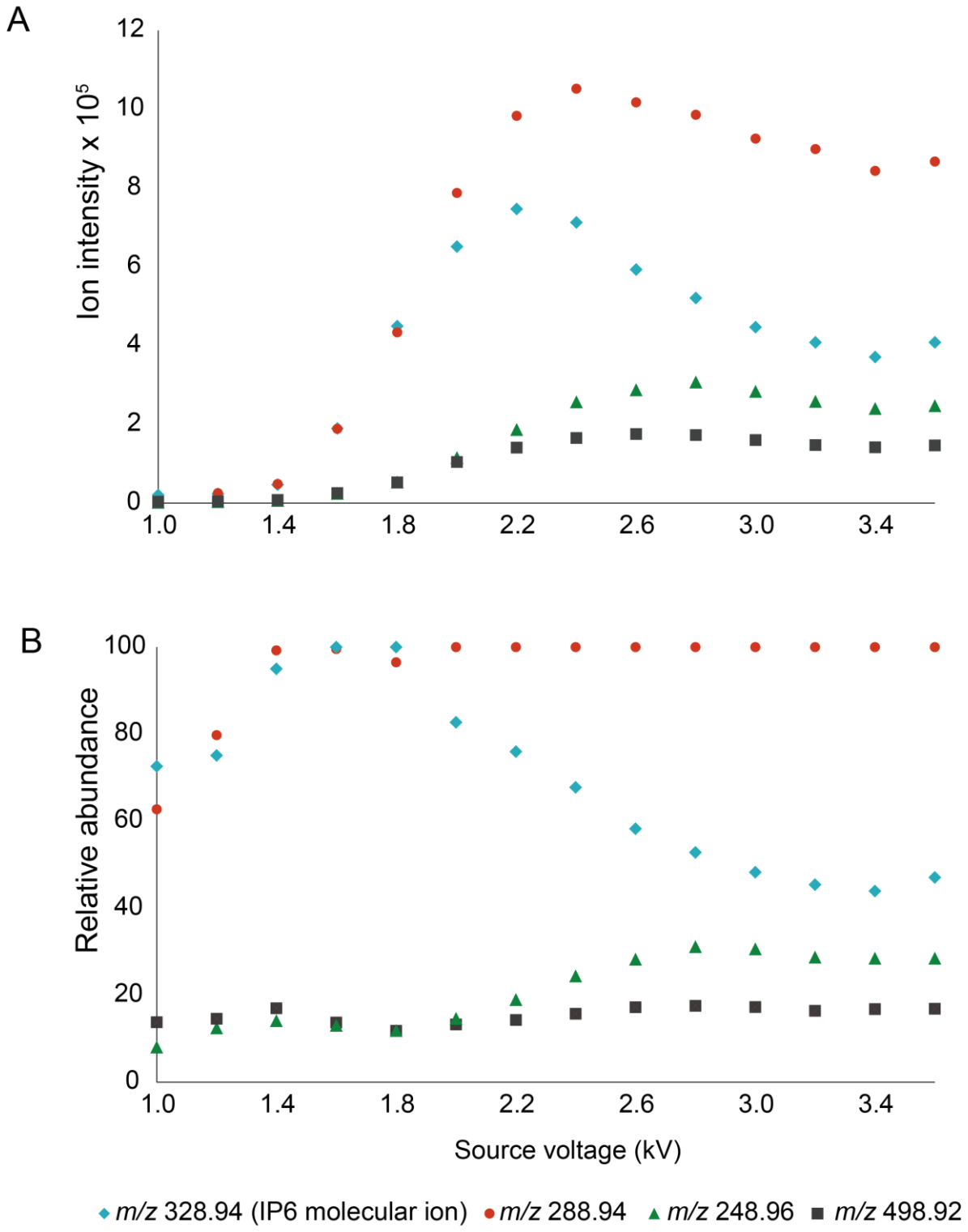


Figure 3.7. The effect of variation of source voltage in negative ion mode on an ESI - Orbitrap Elite versus ion intensity A, and relative abundance B, of key ions in the mass spectrum of IP6.

3.6 Conclusions

Negative ion ESI-Orbitrap mass spectra of IP6 and IP5 reference standards were recorded as part of a wider investigation aimed at incorporating the approach into a new analytical protocol for the assessment of the importance of inositol phosphates and organic P in environmental matrices. The primary findings were:

1. The mass spectra of IP6 and IP5, while complicated, exhibit a characteristic pattern of charge acquisition, fragmentation and formation of adducts of inositol phosphates in the ESI source. Using ion chromatographic purification of the IP6 standard, the isobaric ions in the mass spectra are shown to be due to in-source fragmentation, and not lower homologue inositol phosphate impurities.
2. The loss of water and HPO_3 from the $[M-2H]^{2-}$ ions, and the correlation with the fragmentation patterns seen in the MS/MS experiments support conclusions regarding the mechanisms of fragmentation in the ESI source. Analysis of the changing ion distribution with increasing source voltage, provides further evidence of fragmentation.
3. The use of HRMS increases the certainty of identifications and is crucial for identification of inositol phosphates in complex matrices, such as soil and sediment extracts. From the evidence, it can be concluded that determination of inositol phosphates using ESI-HRMS requires the study of the entire mass spectrum. Ions from in-source fragmentation isobaric with lower inositol phosphates can interfere with MRM experiments, leading to false positive identification of these compounds.

The results of this investigation demonstrate the potential for using full scan ESI-HRMS to study inositol phosphates, with clear gains to be made in incorporating the technique into protocols for the exploration of organic phosphorous cycling in the environment at the molecular level.

Chapter 4. Identification and quantification of IP6 isolated from complex matrices using IC/HRMS and comparison to ^{31}P NMR method.

4.1 Abstract

Analysis of the organic P compound IP6 in soil and manure extracts is often acheived by ^{31}P NMR spectroscopy. This method is time-consuming with questionable accuracy of identification and quantification due to potentially interfering resonances from co-extracted P species. MS offers the opportunity for molecular species identification and the negative ion ESI-HRMS mass spectrum of IP6 has been previously characterised. Combination of ESI-HRMS with IC was applied to the identification and the quantification of IP6 isolated from several complex environmental matrices. Standard NaOH-EDTA extracts of soils and manures were analysed by both IC with ESI-HRMS and ^{31}P NMR and the methods compared. ESI-HRMS analysis of eluate from IC confirms unequivocal identification of IP6. The compound was identified in the NMR *via* overlaying spectra of extracts of soils spiked with IP6. Comparison of quantification *via* standard addition in IC and NMR analysis gave good correlation (r = 0.955). IC with ESI-HRMS was found to be a more sensitive, rapid and reliable method of identification of quantification of IP6 with an LOD of 0.7 mg IP6 kg^{-1} soil and LOQ of 2.1 mg IP6 kg^{-1} soil using IC.

4.2 Introduction

IP6 is an important soil organic P compound. It can constitute up to 50 % of organic P in soils (Murphy *et al.,* 2009) and is thought to provide a source of P to biota in the absence of readily available inorganic phosphate (Turner *et al.*, 2003b). Understanding the role of IP6, and indeed other organic P compounds, in the soil P biogeochemical cycle requires accurate detection and quantification of the compound isolated from the soil matrix. Analysis of individual P compounds in soils is a major challenge due to the complexity and diversity of the material.

IP6 is an organic compound with unusual properties in that it is highly polar and extraction from soil requires strongly basic solution. Inevitably, large quantities of organic and inorganic ions, metals, and natural polymeric substances are coextracted. Analysis methods must overcome interferences from these and contend with the relatively low concentration of P in soils. Molecular level analysis of IP6 using ^{31}P NMR is frustrated by the technical challenge of the limited resolution of the available instrumentation, resulting in low signal-to-noise ratios in spectra of soil extracts. Chromatography, combined with the latest advances in mass spectrometry, offers an alternative method of characterisation of soil organic P compounds.

Currently ^{31}P NMR is a frequently used method for identification and quantification of P in soil extracts. It has the advantage of being P-specific, enabling identification of P compound classes (and individual compounds) as well as quantification. Its application to soil organic P characterisation was first demonstrated in 1980 (Newman & Tate, 1980). In the following decades, NMR has been applied to the quantification of IP6 in a range of soils, manures, and sediments (Giles *et al.*, 2011; Leytem *et al.*, 2006; Carman *et al*., 2000). Identification of the compound is based on the presence of four peaks, theoretically appearing in a 1:2:2:1 ratio (see Figure 4.1), in the monoester region of the spectrum. Quantification may be either by reference to an internal standard, or by determination of the total P of the extract and measurement of the proportion of IP6 in the total spectrum (Turner *et al.*, 2003b).

While ^{31}P NMR is advantageous for the characterisation of organic P compounds in soil extracts to the compound class level (e.g. phosphate monoester, phosphate diester, phosphonate) identification of individual compounds, such as IP6, is not straightforward and often uncertain due to low resolution of peaks and overlapping signals in the spectrum. ^{31}P NMR analyses of soil extracts require long acquisition times (usually overnight) to achieve sufficient signal-to-noise ratios. Difficulties caused by overlapping resonances are particularly

evident in the case of the phosphate monoesters. The monoesters are typically the most abundant soil P compounds observed in the NMR spectrum, but their signals co-occur in a narrow chemical shift range (typically ~ 6-4 ppm). In the absence of spiking with standard compounds, assignment of individual peaks to specific compounds is questionable. Literature values have been reported for the chemical shifts of individual compounds in soil extracts (Turner *et al*., 2003a; Cade-Menun, 2015) and have been referred to in identification of compounds (Turner *et al*., 2014; Reitzel *et al*., 2007; Cade-Menun *et al*., 2015). However, the chemical shift of a compound is reliant on the ionic strength and pH of the solution and values can differ substantially in extracts from those reported.

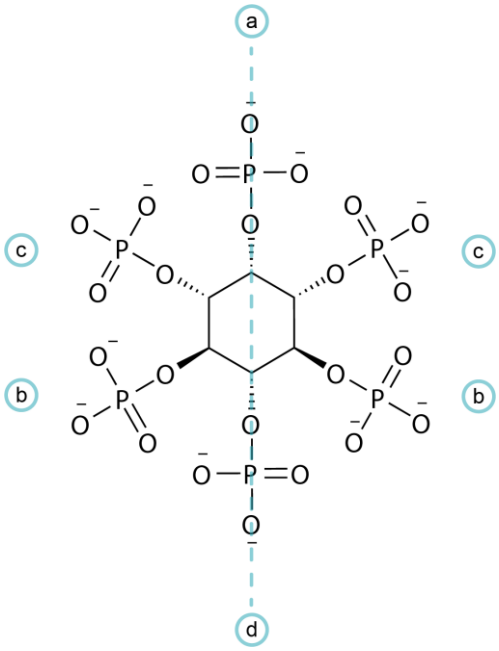


Figure 4.1. IP6 at pH 10-13 with plane of symmetry indicated. Two P nuclei contribute to the b and c signals, leading to peak integrals in a ratio of 1:2:2:1 (Barrientos & Murthy, 1996).

McLaren *et al.* (2015a) identified a broad peak underlying the monoester region in the >10 kDa fraction of soil extracts. Quantification of individual compounds in NMR spectra, once

identified, can be challenging due this underlying P signal. This difficulty is well-known and was addressed by Doolette & Smernik (2015). A range of methodologies were proposed for the deconvolution of NMR spectra. It was suggested that where peaks are fitted and integrated to the baseline (Turner *et al.*, 2003b, see Figure 4.2 A), that the 1:2:2:1 stoichiometry of the signals should be preserved. Individual peaks may be modelled by the spectral analysis software to fit the region and integrated in an effort to simplify the signal (Figure 4.2 B). However, it is widely thought that P signal underlying the monoester region is due to polymeric-P. The deconvolution of the spectrum in this case may be achieved by fitting of a "broad feature" (see Figure 4.2, C) to the region to account for polymeric-P, which is then subtracted prior to integration of peaks. As yet, none of these approaches to spectral deconvolution has been adopted as standard practice.

In NMR spectroscopy, the full signal of a compound is achieved upon complete relaxation of the pulsed nucleus. The T_1 relaxation delay is the time taken for the nucleus to relax to its maximum net magnetization. Ideally, experiments will have relaxation delay times greater than the T_1 of all the nuclei being quantified. For quantification against total P, this requires a relaxation delay that is greater than the longest T_1 of the sample – requiring lengthy experiments to determine the T_1's present. Where an internal standard is used, the chemical environment of the standard P nucleus must be sufficiently similar to that of the analyte P nucleus for meaningful quantification. Differences in the T_1 of the nuclei will result in disproportionate signal from one of the compounds and inaccurate quantification. T_1 relaxation delays are influenced by the chemical environment of the analyte nucleus. For example, paramagnetic ions bound to the analyte reduce the T_1 delay. Soils are an abundant source of paramagnetic ions such as Al, Ca, Mg, Mn, and these inevitably occur in the extract solution

and may therefore have an influence on the T_1 delays of the P nuclei, thereby potentially affecting the quantification of IP6 using this approach.

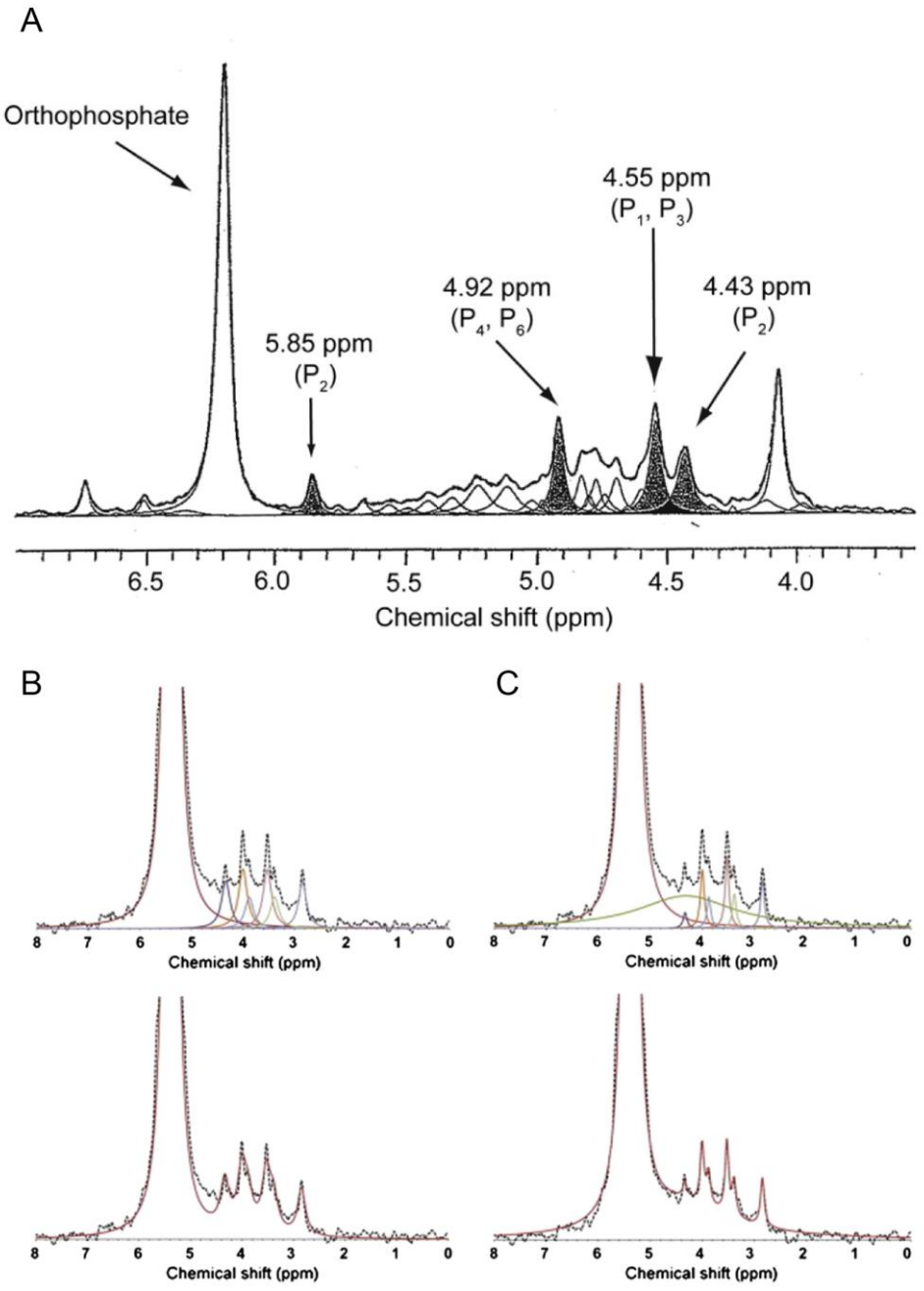


Figure 4.2. The results of different methods of deconvolution of ^{31}P NMR spectrum of a soil NaOH-EDTA extract. In A, fitted peaks are integrated to the absolute baseline. In B seven sharp overlapping peaks are fitted and can be assigned to individual species. In C the fit also includes a broad peak fitted to account for underlying polymeric-P signal. The dotted line represents the actual spectrum, whereas the red line in the lower panels depicts the sum of all the fitted peaks. From Turner *et al.* (2003b), Doolette *et al.* (2011) and Doolette *et al.* (2015).

Chromatography has been applied to the identification of organic P compounds, particularly IPxs, in soil extracts. Leytem *et al.* (2008) extracted P compounds from broiler ileal digesta, litter and manure with HCl, rather than the more frequently used NaOH-EDTA extraction (Turner, 2008) and determined IPx concentrations *via* HPLC with post-column derivatisation with $FeCl_3$. Quantification of IPx and monoester P was compared to NaOH-EDTA extracts analysed by ^{31}P NMR and found to correlate well despite the different extraction procedures used. El-Rifai *et al.* (2008) used size exclusion chromatography (SEC) with selected ion monitoring (SIM) in ESI-MS to identify IP6 in extracts, however, it was not possible to quantify IP6 under these conditions. Sjoberg *et al.* (2016) and Paraskova *et al.* (2015) combined HPLC with MRM mass spectrometry for identification of IPxs in lake sediments. Solid phase extraction (SPE) using a C_{18} cartridge was required to remove matrix effects from "coloured material" which were causing loss of peak resolution between injections and requiring regeneration of the column with offline flushing with base and acid containing solutions to eliminate this effect.

A similar IC approach to that employed in this thesis was used by Waithasong *et al.* (2015), greatly informing the chromatography experiments here. Soil was extracted separately with acid and base. NaOH extracts were acidified with 6 M HCl to precipitate "humic acids" prior to IC analysis. An SPE clean-up step was then introduced for the removal of chloride. Identification and quantification were achieved by retention time and standard addition. The method proposed in this thesis is more straightforward, applying the standard NaOH-EDTA extraction to soils and manures and requiring only filtration and dilution of the extracts prior to chromatographic analysis.

4.3 Aims

The aims of this chapter were:

1. To apply IC and ESI-HRMS to the identification of IP6 in NaOH-EDTA soil and manure extracts.
2. To compare the method of identification and quantification to widely used NMR procedures for three clay soils.
3. To apply the method to the determination of the extraction efficiency of the standard NaOH-EDTA extraction method for IP6 in the selected soils.
4. To compare the use of IC/HRMS as an identification and quantification method to NMR in terms of sensitivity, accuracy, sample preparation and analysis requirements.

4.4 Materials and methods

4.4.1 Soil and manure sampling strategy

Three clay soils under different land uses (arable, grassland and woodland) were collected from within 500 m of each other in the Sem catchment around Prior's Farm in Salisbury. The manures were collected fresh from Prior's Farm (cow), Broadchalke, Salisbury (sheep), Boyton, Warminster (pig) and Chew Valley (chicken). The soils were air dried and sieved to 2 mm prior to extraction, and the manures freeze dried to minimise any risk of infection, and gently crushed.

4.4.2 Extraction

Soils were spiked and extracted as described in Chapter 2, sections 2.3 and 2.5. Manures were extracted without spiking. Each material was extracted in triplicate. Extracts were prepared for IC and NMR as per the methods set out in 2.4. The arable soil freeze dried extracts were analysed *via* NMR in 200 mg mL^{-1} solutions, while the remaining clay soil extracts were analysed at 100 mg mL^{-1}. Total P was determined for each of the soil NaOH-EDTA extracts by method 2.3.4 in section 2.10.4.

4.4.3 Instrumental analysis

IC, ESI-HRMS and NMR analyses were carried out as described in Chapter 2 section 2.10. IC calibration curves of IP6 standard solutions were prepared by the method given in Chapter 2, 2.10.1.2. A cleaning gradient involving blank Milli Q injections was introduced between sample analyses of IP6 to remove carryover.

Integration of the peak identified as IP6 in the ion chromatograms was performed manually using Chromeleon software (see Figure 4.2), taking into account, where necessary, the tailing of the IP6 peak into the neighbouring IP5 peak, as identified in the previous chapter. The calibration curve was used to determine LOD and LOQ as per section 2.10.1.2. Quantification of the IP6 peak was determined initially using the calibration curve, and subsequently using a standard addition approach. In comparing the results, it was found that matrix effects were causing IP6 concentrations to be overestimated in some extracts and underestimated in others. Consequently, a standard addition approach was adopted for all quantifications.

MestreNova software was used for the analysis of NMR spectra as in section 2.10.3.3. The response factor for NMR quantification was determined after an equimolar solution of MDPA and IP6 was analysed under the same conditions as the extract samples. The purity of the IP6 standard was accounted for in the calculations and a response factor for each of the four IP6 peaks in the spectrum was determined and applied to the spectrum integral. Pearson's product moment correlation between quantifications of IP6 by NMR and IC was determined in the statistical package R.

4.5 Results

4.5.1 Ion chromatography of soil and manure extracts

Figure 4.3 depicts the ion chromatograms of extracts of each type of soil (A - C) and the four manures (D - G). The IP6 peak is highlighted in the enlarged section of each chromatogram.

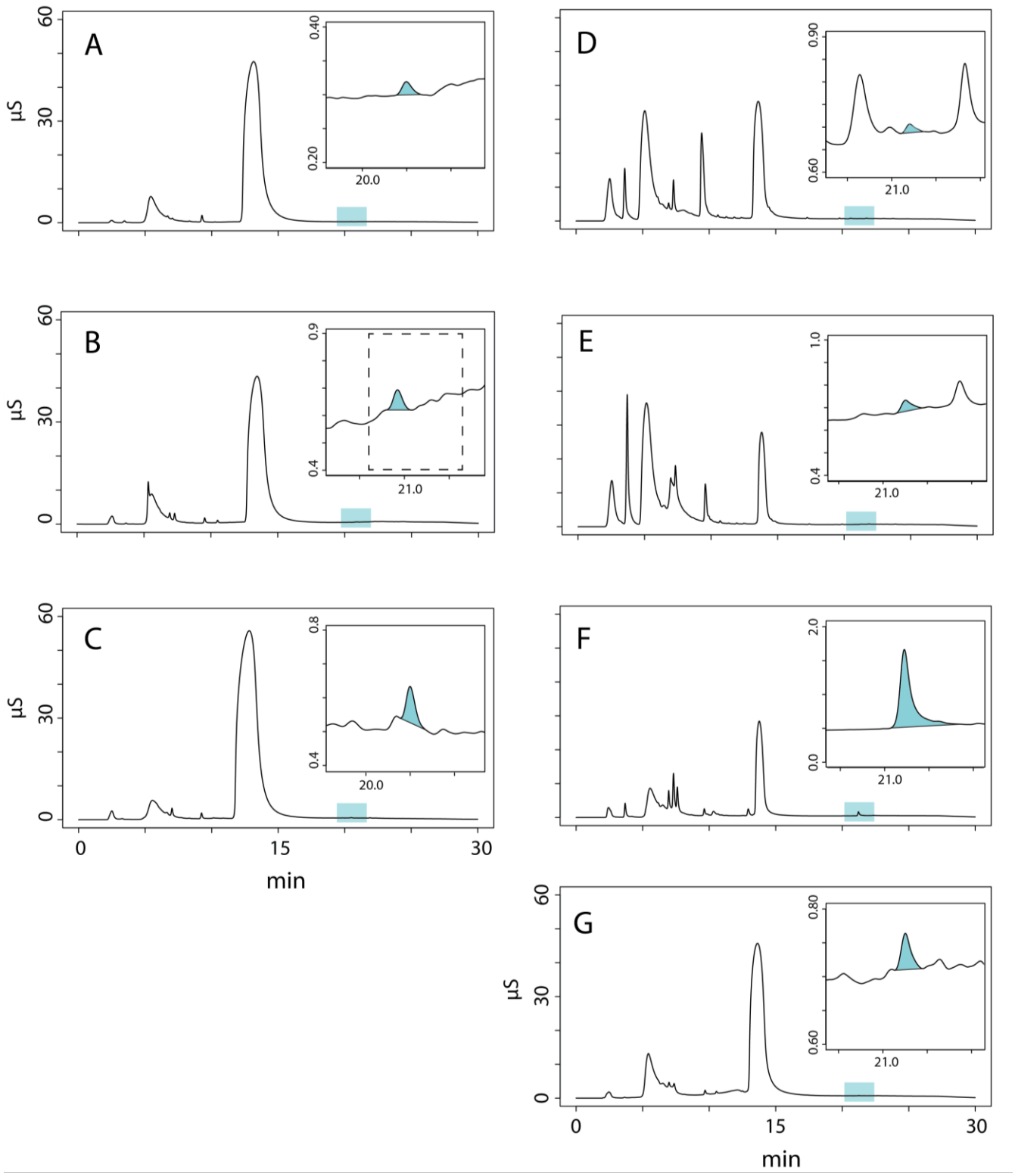


Figure 4.3. Ion chromatograms of soil (A: Arable, B: Grassland, C: Woodland) and manure (D: Sheep, E: Cow, F: Chicken, G: Pig) extracts. The IP6 peak is highlighted in the enlarged segment. Dotted line indicates fraction collected for ESI-HRMS.

In each analysis, the majority of the matrix material elutes in the first 10 min, followed by a large EDTA peak (13 – 15 min), with the IP6 peak appearing at ~ 20 minutes, shortly after the maximum concentration of KOH (70 mM) in the eluent gradient is reached. Peaks eluting immediately after the IP6 peak have been identified as isomers of IP5 in the previous chapter, and the tailing of IP6 into these has been characterised. Due to the higher concentration of IP6 in pig and chicken manure, these extracts were diluted 50-fold, whereas the soil extracts were diluted 20-fold. This enabled a smaller sample load on column for the pig and chicken manure extracts, and therefore minimised impact of the EDTA peak on the chromatogram, as well as reduced the demand for added IP6 reference for the standard addition samples.

A comparison of the 20 to 22-minute region of a control versus spiked grassland soil extract is shown in Figure 4.4 (A). The enhanced IP6 peak is clearly visible in the overlaid chromatogram aiding the identification of the compound. Figure 4.4 (B) depicts the overlaid ion chromatograms of a control soil extract, and the extract following addition of 0.062 µM commercial IP6 reference standard (0.4 ng IP6 per injection). Again, the IP6 peak is visibly enhanced.

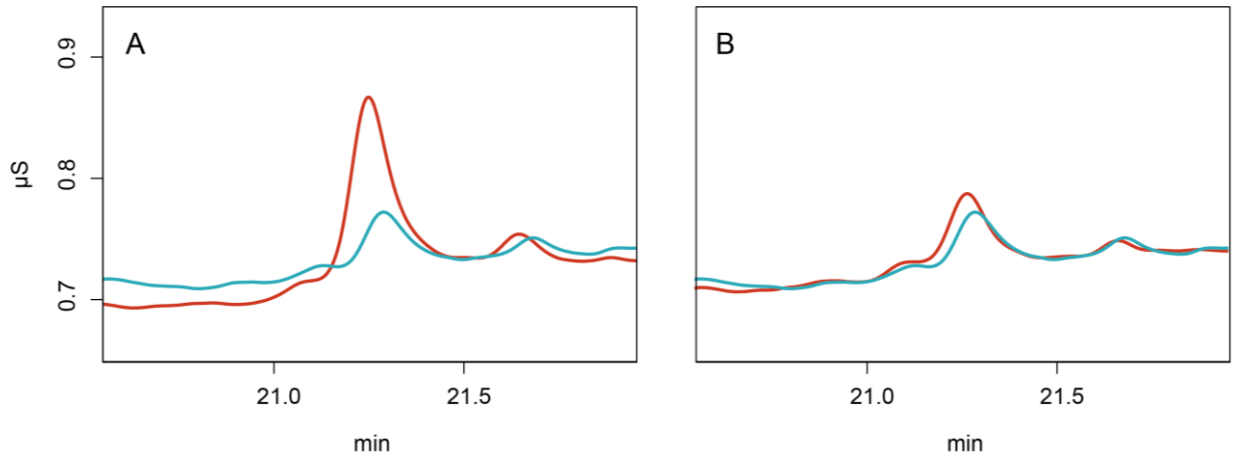


Figure 4.4. Overlaid ion chromatograms of grassland extract. (A) The chromatogram of the control soil extract (blue) overlays the chromatogram of the extract of the soil spiked prior to extraction (red). (B) The chromatogram of the control soil extract overlays the chromatogram of the soil extract including addition of commercial IP6 reference compound (0.4 ng).

4.5.2 HRMS identification of IP6 in IC eluate

The eluate corresponding to the IP6 peak (20.6 – 21.6 min) in the ion chromatogram of the grassland soil (Figure 4.3 B) was collected post-detection and directly infused to the ESI-Orbitrap HRMS. The negative ion mass spectrum of the eluate is presented in Figure 4.5 (A) together with the reference mass spectrum of standard IP6 (B) for comparison and confirmation of the identification. A list of the ions and their identities are given in Table 4.1. The eluate mass spectrum shows the same pattern of multiple charge acquisition, fragmentation and adduct formation as for the reference IP6.

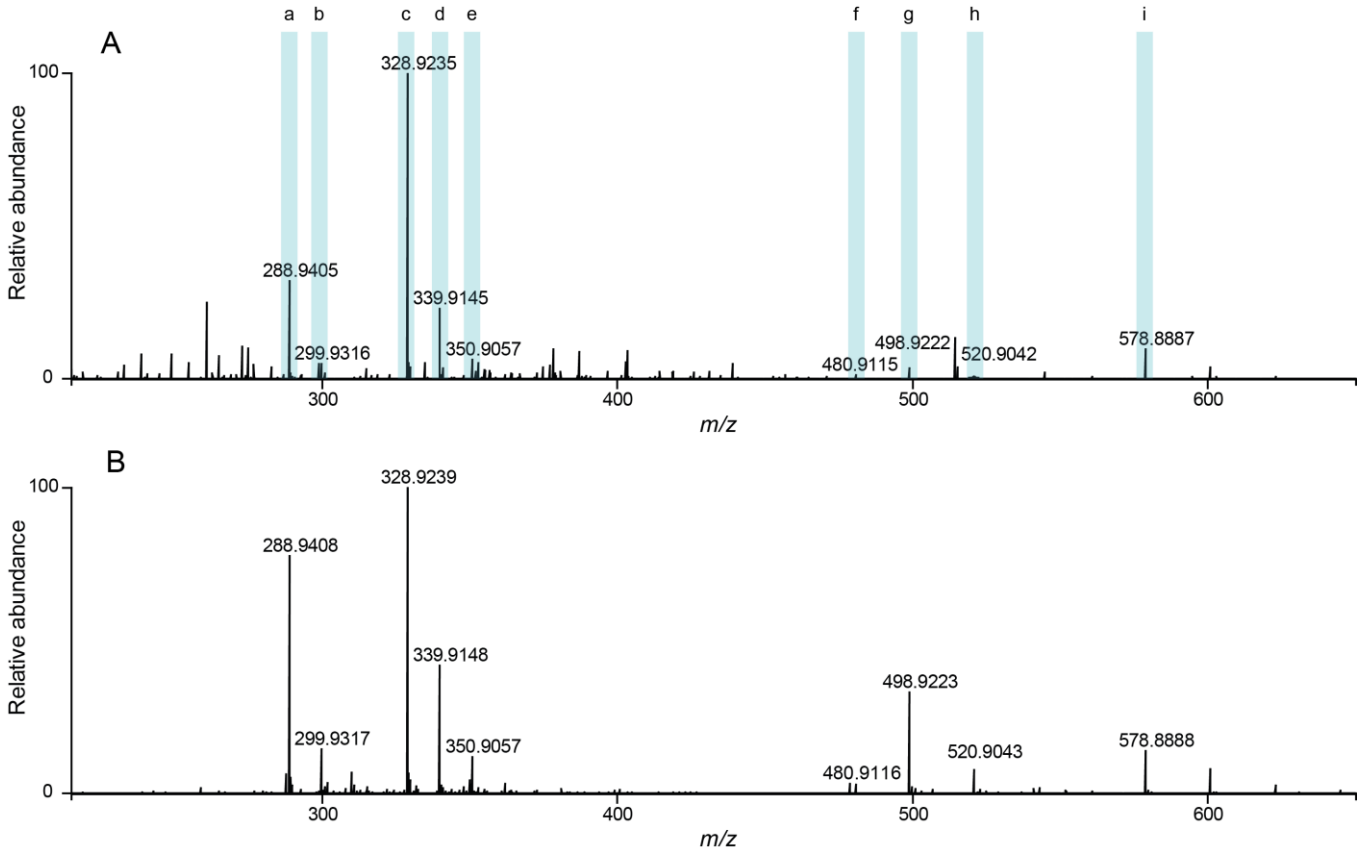


Figure 4.5. ESI-HRMS negative ion mass spectra of isolated eluate from (A) IC and (B) reference IP6 standard. Ions highlighted are identified in Table 4.1.

Table 4.1. Ions identified by ESI-HRMS in Figure 4.4 including formulae and mass measurement errors.

	Ion	z	IP6 soil *m/z*	IP6 ref *m/z*	Formula	Soil IP6 Δ ppm	IP6 ref Δ ppm
a	$[M-HPO_3-2H]^{2-}$	2	288.9405	288.9408	$C_6H_{15}O_{21}P_5$	−1.4	−1.9
b	$[M-HPO_3-3H+Na]^{2-}$	2	299.9316	299.9317	$C_6H_{14}O_{21}P_5Na$	−1.6	−1.7
c	$[M-2H]^{2-}$	2	328.9235	328.9239	$C_6H_{16}O_{24}P_6$	−1.0	−1.6
d	$[M-3H+Na]^{2-}$	2	339.9145	339.9148	$C_6H_{15}O_{24}P_6Na$	−1.0	−1.4
e	$[M-4H+2Na]^{2-}$	2	350.9057	350.9057	$C_6H_{14}O_{24}P_6Na_2$	−1.3	−1.3
f	$[M-2HPO_3-H_2O-H]^{-}$	1	480.9115	480.9116	$C_6H_{13}O_{17}P_4$	−2.5	−2.7
g	$[M-2HPO_3-H]^{-}$	1	498.9222	498.9223	$C_6H_{15}O_{18}P_4$	−2.6	−2.8
h	$[M-2HPO_3-2H+Na]^{-}$	1	520.9042	520.9043	$C_6H_{14}O_{18}P_4Na$	−2.6	−2.8
i	$[M-HPO_3-H]^{-}$	1	578.8887	578.8888	$C_6H_{16}O_{21}P_5$	−2.6	−2.7

4.5.3 Standard addition quantifications

A typical standard addition plot for quantification of IP6 using IC is given in Figure 4.6. Standard addition concentrations were chosen after an initial approximate quantification against the external calibration graph. Calculations were made to determine the IP6 concentration required to enhance peak area by a clearly perceptible amount while minimising any matrix effects caused by the addition of the standard. The overlaid ion chromatograms of the grassland extract demonstrating the peak enhancement are shown in Figure 4.4. The impurity of the standard, as identified in the previous chapter was taken into account in the calculation of concentrations. The form of the ion chromatograms did not appear to be affected by the addition and the range of R^2 values for the standard addition plots was 0.9269 – 0.9996. It was concluded that the standard addition did not cause any deleterious effects and that SPE clean-up was not required.

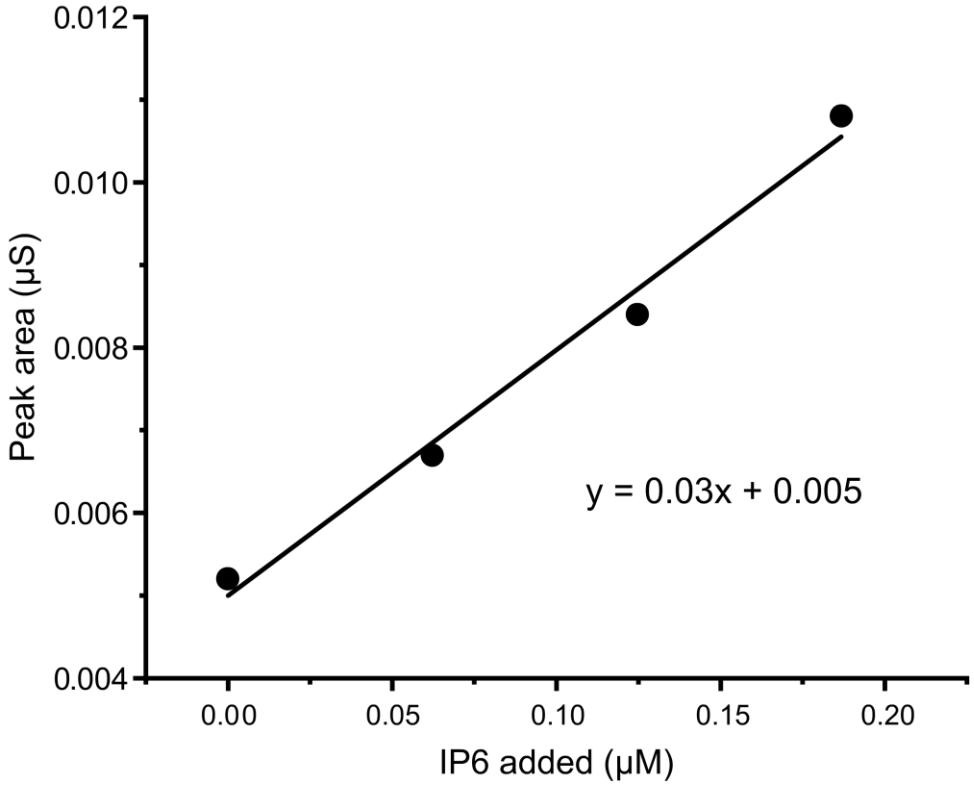


Figure 4.6. Standard addition plot for quantification of IP6 in grassland control soil extract. The R^2 (0.9876) indicates a good fit of the data to a straight line and that matrix effects were not observed at this level of standard addition.

Figure 4.7 shows the overlaid full ^{31}P NMR spectra of the soil extracts of both control and spiked soils of each land use type. The internal standard (MDPA), the monoester-P, the diester-P, and pyrophosphate regions are indicated. The monoester region (6 – 2 ppm) is enlarged to show the detail. The form of the control and spiked extract spectra is almost identical for each soil type, except for the IP6 peaks. The identification of the four IP6 resonances (chemical shifts: IP6a 5.75, IP6b 4.81, IP6c 4.44 and IP6d 4.33 ppm) is verified by the enhanced peak height in the spiked samples. IP6 was not detected in one of the woodland control soil extracts and none of the arable control extracts, even at 200 mg mL^{-1} concentration. The chemical shifts in the NMR spectra of the 200 mg mL^{-1} spiked arable soil extracts were altered by up to − 0.06 ppm compared to 100 mg mL^{-1} extracts.

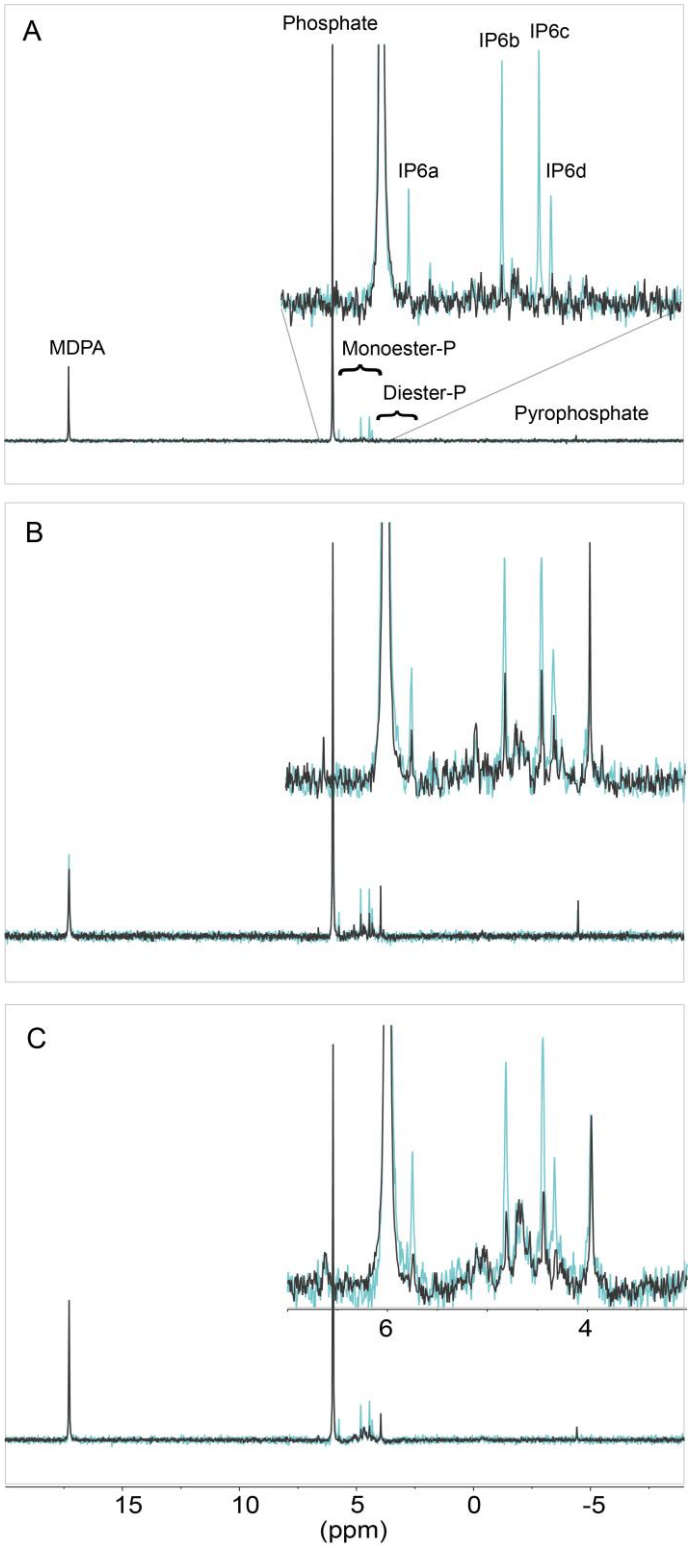


Figure 4.7. Overlaid NMR spectra of soil extracts (A: Arable, B: Grassland, C: Woodland) with control soil extracts in grey and spiked extracts in blue. Internal standard (MDPA), orthophosphate, monoester-P, diester-P and pyrophosphate regions are indicated. The monoester region (6 – 4 ppm) is enlarged in each. IP6 resonances are identified by the four enhanced peaks in the enlarged region as labelled in A.

4.5.4 Identification and quantification of IP6 based on NMR spectra

Quantification of IP6 in the NMR spectrum was calculated after integration of the MDPA and the four IP6 peaks. The value for each of the IP6 peaks was adjusted using the response factor calculated on the equimolar solution of MDPA and IP6 reference compounds. The peak areas of the four IP6 resonances were not consistently observed in the expected ratio of 1:2:2:1, indicating underlying signals in this critical region. Therefore, the lowest peak area of the four was chosen for the basis of the quantification, as it was assumed that this peak was least influenced by underlying signals. This was one of the methods proposed by Doolette & Smernik (2015). Almost no monoester P was detected in the control arable soil extract, and the IP6 peak ratios in the spiked extract NMR spectrum are closest to 1:2:2:1 of the three soil types.

4.5.5 Quantification, extraction efficiency, and comparison of methods

The concentrations of IP6 in each of the soil extracts, as determined by IC and NMR, the extraction efficiency of NaOH-EDTA for IP6, and the IC LOD and LOQ are presented in Table 4.2. IP6 concentration was highest in the pig and chicken manures. The woodland soil was found to have the highest concentration of IP6 by IC. The control arable soil had concentrations above the LOD, but below the LOQ for IC. Extraction efficiency of NaOH-EDTA for IP6 from the soils, as determined by IC, ranged from 62.1 – 69.4 %. The woodland soil had the lowest extraction efficiency, and the arable soil the highest.

Table 4.2. IP6 concentrations as determined by IC and NMR and limit of detection (LOD) and limit of quantification (LOQ) of IC. *C = control, S = spiked.*

Soil Extract	IP6 by IC	Average IP6	Extraction efficiency	IP6 by NMR	Average IP6	Extraction efficiency
	mg kg^{-1}		%	mg kg^{-1}	mg kg^{-1}	%
Arable C1	10.09			ND		
Arable C2	4.32	7.2 ± 2.9		ND		
Arable C3	7.12			ND		
Arable S1	199.54			182.75		
Arable S2	128.19	164.9 ± 35.7	69.4	142.59	159.5 ± 20.8	
Arable S3	167.01			153.01		
Grassland C1	79.42			74.42		
Grassland C2	32.92	52.8 ± 24.0		33.74	55.9 ± 20.6	
Grassland C3	45.99			59.47		
Grassland S1	124.54			171.49		
Grassland S2	233.9	188.9 ± 57.2	66.7	230.41	221.6 ± 46.3	77.4
Grassland S3	208.22			262.74		
Woodland C1	51.55			ND		
Woodland C2	68.55	61.2 ± 8.7		49.73	52.3 ± 3.7	
Woodland C3	63.48			54.95		
Woodland S1	248.8			239.79		
Woodland S2	138.24	181.0 ± 59.4	62.1	156.67	186.2 ± 46.5	65.8
Woodland S3	156.04			162.24		
LOD	0.7					
LOQ	2.1					

Figure 4.8 depicts the correlation of the quantification of IP6 by both IC and NMR where IP6 was detected by both methods for each sample. The slope of the fitted line is 0.9456, indicating an almost 1:1 relationship. The Pearson correlation coefficient was found to be 0.955 with $P < 0.001$.

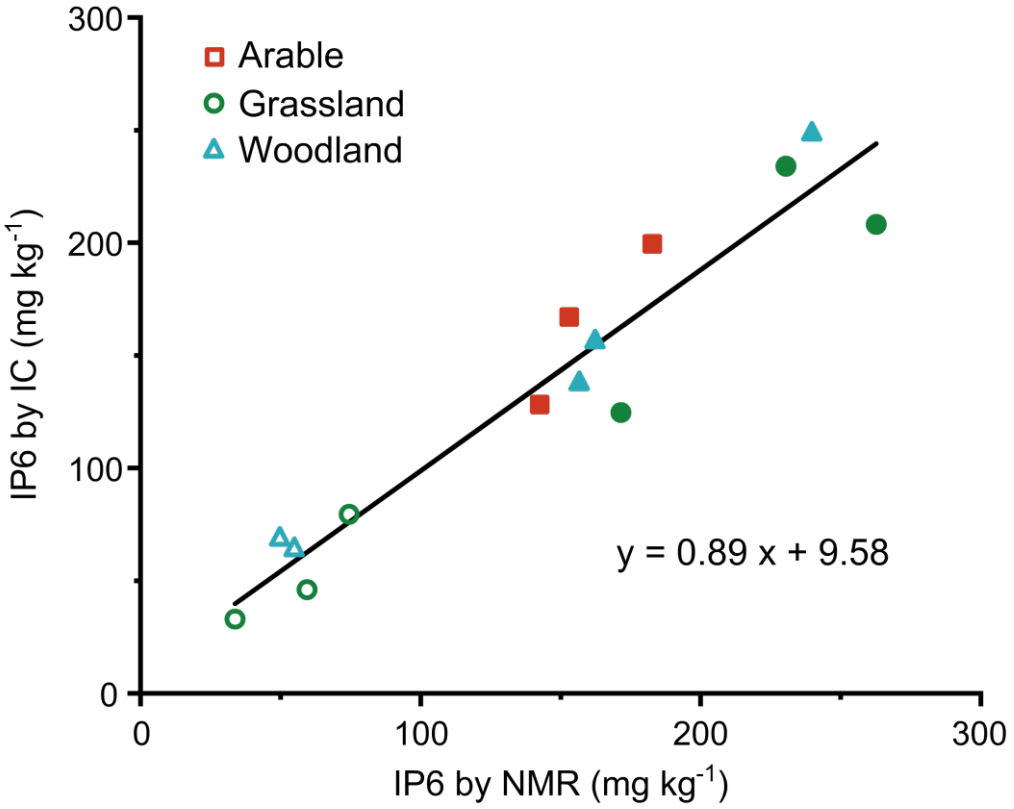


Figure 4.8. Plot of IP6 concentrations determined by IC and NMR and Pearson's product moment correlation ($r = 0.9554$, $p \leq 0.001$). Control = open shape, spiked = filled shape.

4.6 Discussion

4.6.1 Use of IC for IP6 analysis

The ion chromatograms of the soil and manure extracts shows good separation of IP6 from the matrix material. The IP6 elutes sufficiently late in the chromatogram to prevent interference from the tailing EDTA peak. The pig, sheep, and to a lesser extent cow, manure extract chromatograms display peaks (as yet unidentified) in the same region as the IP6 peak. The majority of the matrix material elutes in the first 10 min and varies in form and quantity between extracts. Exploring the nature and diversity of the compounds in the matrix material was beyond the scope of this study, but clearly this material has potential for further investigation using this method.

4.6.2 ESI-HRMS identification of IP6

The ESI-HRMS negative ion mass spectrum of the trapped eluate is identical to that of the commercial IP6 reference compound (Figure 4.5). The characteristic behaviour of IP6 in ESI-HRMS, and the ions generated in the mass spectrum, have been discussed comprehensively in McIntyre *et al.* (2017) and Chapter 3. The ESI-HRMS spectra provide unequivocal confirmation of the identification of IP6 isolated from the soil matrix. In preliminary experiments (see Chapter 8), IP6 was not identified in a soil extract mass spectrum even when spiked directly into the extract. This was likely due to the high ionic strength of the extract, caused by the high concentrations of hydroxide ion, metals and salts in the extractant and vast range of other charged species occurring in soils. Competition between these ions in the ESI source cause clustering (Zhou & Hamburger, 1996) and ion suppression of analytes of interest (King *et al.,* 2000).

The advantages of using IC to isolate IP6 are twofold. First, the chromatographic separation of compounds from the matrix material, provides a purified sample with fewer interfering compounds for ESI-HRMS. Second, the ion suppressor in the IC instrument removes K^+ ions from the eluate *via* cation exchange for H^+ ions across a membrane. Coupled with the reduction, or indeed elimination, of matrix compounds *via* chromatography, the ion suppression results in a predominantly aqueous solution containing the IP6 analyte. Unhindered by competition from salts in solution in the ESI, ionisation of the analyte is achieved, enabling analysis of the compound and unambiguous identification.

4.6.3 NMR analysis

The chemical shift of the four IP6 peaks were found by the overlaying of spectra for the same soil types and identification of the peaks enhanced between the control and spiked soil extracts. The chemical shifts were within a 0.02 ppm window for the 100 mg mL^{-1} spectra but shifted

up to 0.06 ppm in the 200 mg mL^{-1} spectra. This was likely due to the difference in ionic strength of the higher concentration solutions and illustrates the difficulty in relying on literature values for identification of individual compounds in soil ^{31}P NMR.

The low concentration of organic P in the arable extracts is evident by the lack of signal in the monoester- and diester-P regions of the NMR spectrum. The spectrum is dominated by the PO_4^{3-} peak. A low abundance of pyrophosphate is observed around −4.5 ppm. The grassland soil extracts contained more monoester P, and the highest concentration of pyrophosphate of the three soil types. The large peak observed at 3.92 ppm in the grassland and woodland extract spectra could potentially be β-glycerophosphate according to the Turner *et al.* (2004), or *scyllo*-inositol hexakisphosphate according to Jørgensen *et al.* (2011) literature values but would require spiking with standard to confirm the assignment. Monoester P was most abundant in the woodland soil extracts, as well as some pyrophosphate signal at -4.5 ppm.

4.6.4 Quantification of IP6

Quantification of IP6 using standard addition in IC shows that IP6 concentration was highest in the pig and chicken manures reflecting the high grain content of their diets, particularly the chickens. Poultry and pigs are less capable than ruminants of digesting the IP6 in their feed and subsequently have higher concentrations of the compound in their manure (Hill *et al.,* 2008, Woyengo & Nyachoti, 2013). The sheep and cows were grass fed and had lower concentrations of IP6 in their manures than the grain fed animals. Quantifications were in line with literature reports of IP6 concentrations in soils and manures (Giles *et al.,* 2011, He *et al.,* 2006, Ray *et al*., 2012).

Using the response factor, quantification results from NMR were in good agreement to those of IC for the majority of samples (Figure 4.7), suggesting that quantification using both methods is comparable. However, no IP6 was detected in the arable control NMR spectra,

despite doubling the concentration of the samples to 200 mg mL^{-1}. The arable soil extract NMR spectra, despite having similar TP concentrations to the other soil extracts, contained very little organic P, but did have a much larger proportion of PO_4^{3-} as evidenced by the large peak in the NMR spectra. The low organic P content resulted in an almost 1:2:2:1 ratio for the IP6 peaks in the spiked samples. The integration of these peaks was therefore determined to be least affected by underlying broad P signal in the spectrum. This supports the conclusion that the use of lowest peak area in this experiment was the best quantitative approach and that variation in peak ratio is caused by underlying interferences such as the broad feature depicted in Figure 4.1.

Determination of the response factor for quantification of IP6 relative to MDPA was a faster and more practical alternative to measurement of T_1 values for the IP6 peaks which requires multiple lengthy experiments and calculations (Zhang *et al*., 1998). The response factor method appears to be reasonably accurate given the agreement in quantification with that determined by IC. The T_1 relaxation of a nucleus may be influenced by paramagnetic ions in the sample solution if they are in sufficiently high concentration and bound to the analyte of interest. We are confident there is no interference in the quantification from paramagnetic ions for two reasons. Firstly, while the paramagnetic ion concentration of a soil extract solution will be high due to co-extracted metal ions, the EDTA in the re-dissolved extract solution will prevent binding to the IP6. Secondly, the effect of the paramagnetic ions would be to shorten the T_1 relaxation of the IP6 nuclei, and the use of the response factor in this situation would cause overestimation of the IP6 concentration. Since the quantification performed using the response factor correlated very well with that calculated *via* standard addition in IC, it was concluded that the response factor method of quantification using the lowest peak area was a reliable approach.

The extraction efficiencies of NaOH-EDTA for IP6, determined by spiking the soil prior to extraction, given in Table 4.2 are complete for the IC data, but not for NMR quantification data. They are in the range of 62 – 69 %, however, the reasons for the differences in extraction efficiencies between soils and the fact that recovery is not complete are not clear from these data and are explored further in Chapter 5.

The standard deviations of the IP6 concentrations determined are in the range of 7 to 45 %. Similar variances are observed for the same soil types using each method. Individual replicate IP6 concentrations are in good agreement determined *via* IC or NMR as evidenced in Figure 4.8. The variance observed, therefore, is a product of the extract procedure. This suggested discussion on how to better homogenise soil samples for extraction and/or increase replicate numbers is required for future studies.

4.6.5 Method comparison: IC v NMR

Analysis of the quantification of each extract using both methods *via* the Pearson's product moment, gives good correlation between the methods and an almost 1:1 relationship between the concentrations that were measured, validating the approaches adopted.

Quantification of IP6 using standard addition in IC was found to be more rapid than quantification using NMR, even including time taken for the cleaning gradient between sample injections to remove carry over. The total instrument time required per extract analysis in IC is 6 h, while each NMR analysis takes 16 h, making IC analysis 2.5 times faster than NMR. Additionally, sample preparation is much quicker and more straightforward for IC. Extracts are syringe filtered and diluted for standard addition in IC (ca. 30 min), while extracts for NMR must be frozen (12 h), and freeze-dried (~ 36 – 48 h), before being homogenised, weighed, re-dissolved and centrifuged (total = 1 h). Sample requirements for IC are also far lower than for NMR. Each NMR analysis required at least 100 mg of freeze-dried powder, which equates to

about 5 mL of extract solution. Each IC analysis required 200 µL (50 µL × 4) of extract solution for full quantitative results. The detection and quantification ability of IC (LOD = 0.7 mg kg^{-1} soil, LOQ = 2.1 mg kg^{-1} soil) cannot be matched by NMR.

Quantification of IP6 in individual extracts and standard deviations of the quantifications (Table 4.2) are similar between methods. The variances seen in the quantification are therefore variances in the extraction procedure of the 1.5 g replicates using this method. The improvement of homogenisation of the soil samples and extraction will be important in future studies using these soils.

4.7 Conclusions

This work demonstrates the development of a novel and robust method for the identification and quantification of the soil organic P compound, IP6 extracted from soil matrices. It overcomes the difficulties encountered in NMR due to low signal-to-noise of spectra, and in HRMS due to complex and strongly ionic soil extract solutions.

1. The use of IC/HRMS for identification and quantification of IP6 in soil and manure extracts is demonstrated and shown to be advantageous for these complex matrices. The IP6 isolated by IC is free from significant interferences allowing the use of ESI-HRMS, with comparison to a standard reference mass spectrum, to make unequivocal identifications of the target compound. This contrasts to the difficulties encountered in NMR spectroscopy, particularly in the absence of spiking, where IP6 identification and quantification may be confounded by different chemical shifts, overlapping peaks or underlying signals.
2. The ionisation of the IP6 in the eluate collected is facilitated by the ion suppression component of the ion chromatograph. The removal of salt contaminants eliminates

signal dispersion in the ESI caused by ion suppression and competition between matrix ions.

3. The standard addition method of quantification was found to be necessary as quantification with reference to an external calibration was impaired by matrix effects, which were unpredictable. Standard addition plots gave good R^2 values. The LOD and LOQ of IP6 in IC were found to be 0.7 and 2.1 mg kg^{-1} soil, respectively. A cleaning program was required between samples to remove residual IP6 from the system. Nevertheless, the standard addition in IC method of quantification was much quicker than NMR in terms of sample preparation and analysis time. A much smaller amount of extract was required for each IC (200 µL) than for NMR (5 mL) analysis.
4. Quantification of soil IP6 using both NMR and IC correlated well ($r = 0.955$) indicating that the methods were comparable. Standard deviations for replicate analyses of extracts of different sample type were similar between IC and NMR. Therefore, variance seen in the quantitation between replicates was due to variance in either the homogenisation of the soil sample or inherent in the extraction procedure itself.
5. The extraction efficiency of the NaOH-EDTA extraction method for IP6 from soils was briefly investigated. As expected, extraction efficiency results from NMR and IC were broadly similar where IP6 was detected in both control and spiked samples. The reasons for the difference in extraction efficiency between soil types was not immediately apparent and it was decided to further investigate this in the following chapter.

Together, these results show that the IC quantification method is more rapid and more sensitive than NMR, with a lower LOD, and concentration results have similar precision. Standard addition in IC can be used in place of NMR for quantification of IP6, and offline ESI-HRMS can be used to unequivocally confirm the identification of IP6 extracted from complex matrices. The sensitivity, rapidity and low sample requirements of the IC

quantification may be exploited, and multiple replicate analyses performed for different extracts, enabling more complex ambitious experiments to be undertaken leading to more rigorous or comprehensive assessments being obtained.

Chapter 5. Determination of the efficiency of the NaOH-EDTA extraction method for IP6 in relation to soil properties.

5.1 Abstract

Understanding the nature of the binding and retention of organic P in soils is important for management of soil P content for agriculture and control of P transfer to water. The strength of extraction solutions can be used to infer the strength of soil-P binding and differences in availability for plant uptake of the extracted P pools. IP6 is a major soil organic P compound and, has been found through sorption studies to sorb strongly to soil particles, particularly minerals, making it resistant to biodegradation resulting in accumulation in soils. A survey of the IP6 concentration of a range of soil types and land covers was undertaken. Soils were spiked with IP6 prior to extraction to determine the efficiency of the NaOH-EDTA extraction method for IP6 (EEIP6). Soil properties, including metal concentrations and organic content (LOI) were measured and Pearson's product moment correlation analysis was performed to determine the relationship between soil characteristics and EEIP6. Significant positive correlations were found between EEIP6 and total metal concentration ($r = 0.8472$, $p = 0.0039$) and soil Fe concentration ($r = 0.7259$, $p = 0.0268$). A significant negative correlation was found between EEIP6 and LOI ($r = -0.8039$, $p = 0.0090$). The same relationships were not observed for total P extraction efficiency (EETP). These results suggest that although IP6 may sorb readily to the mineral fraction of soils, it is not necessarily strongly retained as soils with high total metal concentrations and low organic content have highest EEIP6. It should therefore be noted that using the NaOH-EDTA extraction for these soils may overestimate IP6 concentrations relative to total P. The detailed analysis undertaken here reveals trends in behaviour of IP6 and total P that diverge in different soil conditions and reinforce the argument that molecular level understanding of the P biogeochemical cycle is required for management of the human influence on the cycle.

5.2 Introduction

The availability of P for plant uptake from soils and its potential for transport to water courses is in part governed by its binding to, and retention on, soil particles. P compounds must be desorbed to the soil solution before mineralisation and uptake by biota or transportation in the dissolved fraction but may be physically protected by aggregation and sorption to soil material from enzymatic hydrolysis (Jarosch *et al*., 2015). Inorganic and organic P compounds bind to varying degrees to soil and this is influenced by the chemistry of those compounds and by a wide range of interacting soil characteristics, such as pH, mineral composition, organic content to name a few. Understanding the nature of soil-P interactions has long been a subject of inquiry and helps inform management practices for soil fertilisation for agriculture and prevention of transport to water.

Sorption and fractionation studies are used to infer binding, accumulation and availability of total P and individual P species to soils. Chang & Jackson (1957) developed a fractionation protocol to extract soil inorganic phosphate in occluded and salt forms of aluminium phosphate, iron phosphate, and calcium phosphate. The P extracted from soils by the solutions at each step have since become operationally defined Al-P, Fe-P and Ca-P in many studies of soil P fractions. Hedley *et al.* (1982) and Tiessen *et al.* (1984) developed fractionation protocols using solutions with differing chemical properties to extract chemical "pools" of P compounds from soils. The pools are operationally defined with decreasing availability to biota related to increasing strength of extraction solution. For example, the Olsen P test (Olsen *et al.*, 1954) extracts P from soils using a bicarbonate solution. It is inferred that the P extracted by this relatively mild solution correlates to bioavailable P and the Olsen P soil test is recommended for calculation of application rates of P fertiliser in Denmark, England, France, Italy and Spain (Jordan-Meille *et al.,* 2012).

The sorption of P and IP6 to soils, particularly to the minerals in soils, has been studied in some detail, *via* addition of compounds to soil or mineral solutions and fitting of a Langmuir isotherm (for example Olsen *et al.,* 1957, Fink *et al*., 2015). Organic P, particularly monoester P, is found to correlate with Al and Fe soil surfaces (Stutter *et al*., 2015; Ohno & Zibilske, 2011), and may therefore accumulate in association with these metals in soils. Much of this work studies the sorption of PO_4^{3-} in solution on to mineral phases, but there are also studies on the sorption of IP6: it is hypothesised, for example, that IP6 binds strongly to metal cations in soils, more strongly than PO_4^{3-} (McKercher & Anderson, 1989), and to "humics" *via* Al- or Fe- bridges (Borie & Zunino, 1983), and as such is immobilised and accumulates in soils. The exact mechanisms of binding are yet to be understood although it is expected to involve binding to the latter cations *via* the oxygens of the phosphate group (Figure 5.1), and that the more phosphate groups on a compound, the tighter will be the binding (Ognalaga *et al*., 1994).

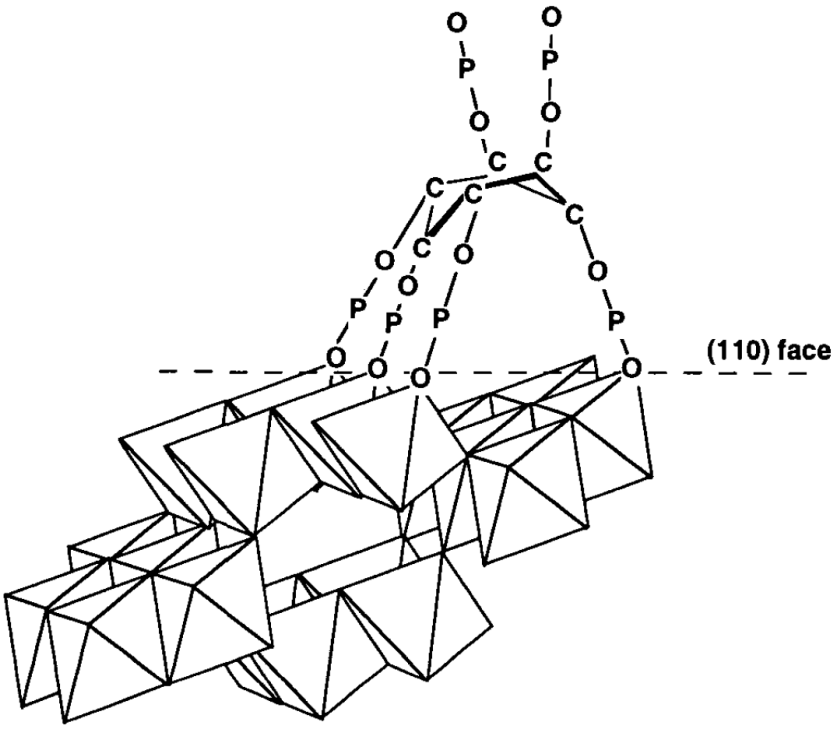


Figure 5.1. Proposed conformation of IP6 sorption to the surface of goethite. For the sake of simplicity, the H atoms and OH groups of the phosphate groups were omitted. From (Ognalaga *et al.,* 1994)

Since Bowman & Moir's (1983) combination of EDTA with NaOH for extraction of soil organic P, the solution has been used extensively for characterisation of soil, sediment and manure P *via* NMR spectroscopy. There has been little variation in the extraction conditions used over the intervening decades, but some studies have investigated alterations to the ratio of sample mass to solution volume, the optimum extraction time or temperature, or varied the concentrations of the extractants. For example, McLaren *et al.* (2015b) found that increasing the soil to solution ratio from the more commonly used 1:20 to 1:4 *w/v* led to better spectral sensitivity in the analysis of low organic P pasture soils *via* NMR. Such variations are intended to enhance the extraction of organic P from materials with different characteristics. The most frequently used parameters are 0.25 M NaOH/ 0.05 M EDTA, in a 1:20 soil to solution ratio, with 4 h extraction at ambient temperature, as recommended by Turner (2008).

In the literature, the extraction efficiency of NaOH-EDTA for total P (EETP) from soils has frequently been included in studies of soil organic P by determination of total P concentration in the NaOH-EDTA extract solution and expressing it as a percentage of the total soil P determined after digestion of soil with concentrated acid, such as aqua regia. The results are varied, for example efficiencies reported by Turner *et al.* (2003c) ranged from 45 – 88 % for twenty-nine permanent pasture soils in England and Wales. In Turner (2008), the range of extractant concentrations (NaOH: 0.05 to 1.0 M, EDTA: 0, 0.025 or 0.050 M) tested on a tropical forest soil yielded at most 46.2 % of total P at concentrations of 0.50 M NaOH and 0.05 M EDTA. Cade-Menun *et al.* (2015) achieved extraction efficiencies of 63 – 83 % in a Cecil (sandy loam) soil. Low recovery of P in manures has been attributed to the formation of insoluble Fe-P and Al-P compounds (McDowell & Stewart, 2005). While the performance of the NaOH-EDTA extraction varies between materials and does not always quantitatively extract P, it is the most efficient one-step extraction method, extracting the highest proportion

of total P and the greatest diversity of P compounds versus Chelex chelating resin and NaOH, Chelex in water, and NaOH alone (Cade-Menun & Preston, 1996).

The EEIP6 using a standard NaOH-EDTA extraction protocol has not been studied at all in soils, but individual studies have determined EEIP6 for individual natural matrices. For example, Ray *et al*. (2012) investigated the recovery of IP6, added prior to extraction, from feeds and faeces of dairy cattle using HCl and NaOH-EDTA, finding that NaOH-EDTA was a better extractant with recoveries up to 105.5 % in faeces. Quantification of IP6 in extracts was *via* HPIC with post-column derivatization. A subsequent study by Rippner *et al.* (2014) studied the EEIP6 from dairy manure and boiler litter in the presence of Al and Fe. It was found that recovery decreased with increasing Al concentration, although was 100 % at typical Al concentrations. The exact method of spiking of IP6 (and Al and Fe) is not clear, however. In the absence of studies of EEIP6 on soils spiked prior to extraction, the underlying mechanisms governing IP6 extraction and total P extraction, and the differences between them, have not so far been determined using an extensive survey of soil types.

The characteristics of soil type, land cover and land management all intersect to contribute to the binding of P to soils. Soil systems are complex and disentangling the influence of individual soil properties is not straightforward. The binding of P to soils under certain conditions may relate to accumulation of some forms of P. However, the ability of plants to retrieve P compounds from soil, and the accumulation of P in soils are not necessarily related to the physical sorption factors or to the extraction conditions alone. Liu *et al*. (2014) found that crop uptake depleted (operationally defined) Al-P and Fe-P pools after cessation of long-term fertilisation of agricultural fields. It is clear that determining the availability of P for biotic uptake and its binding to soil is influenced by multiple intersecting factors including the sorption properties of the soil, the ease of extraction of the P compounds under different

conditions, and the correlation of the extraction with P acquisition strategies of the various groups of the soil biota.

5.3 Aims

The aims of this chapter were:

1. To apply the method of identification and quantification of IP6 developed in Chapter 4 to a detailed study of the EEIP6 of a range of different soil types *via* spiking with IP6 prior to extraction.
2. To quantify the IP6 concentrations of three soil types under three different land covers.
3. Determine the properties of the soils that may relate to soil-IP6 binding and to correlate those properties with the EEIP6.
4. To compare the soil characteristics that influence EEIP6 with those that influence EETP.

In this way we can begin to unravel the factors that influence IP6 binding to soil and the strength of retention of IP6 in soils under extraction conditions.

5.4 Methodology

Soils were collected from three locations in South West England under three land covers (Figures 5.2 and 5.3). The soil types were: clay, alluvial and peat. The clay soil (Prior's Farm – PF) is classified by the Land Information System (Hallett *et al.,* 2017) as slowly permeable, seasonally wet, slightly acid, but base-rich loamy and clayey. The alluvial soil (Quidhampton – QH) is a loamy and clayey floodplain soil with naturally high groundwater. The peat (Somerset Levels – SL) is a fen peat, naturally lime-rich. The land covers in each soil type were: arable (AR), grassland (GR) and woodland (WD). The soil types and land covers (3 x 3) totalled 9 different soil sampling locations. The clay grassland was improved grassland grazed by cattle, whereas the alluvial and peat grasslands were unimproved and ungrazed. The soils

were chosen to represent a broad variety of properties within the region of the South West England. Soil types and land covers were chosen to reflect diverse inputs and influences on soil physical and chemical properties.

Grass and leaf litter were removed, and soils were collected from the top 10 cm in a furnaced glass jar. Soils were air dried until constant mass at room temperature and then sieved to 2 mm.

Soils were spiked prior to extraction and both spiked and control soils extracted in triplicate as per the method in Section 2.3. IC was performed on the extracts as per the method section 2.10.1. Total P of NaOH-EDTA extracts was determined as per section 2.10.4. Bulk soil metal and P determination was as per section 2.10.6, LOI as per 2.10.7 and soil pH as per section 2.10.8. T-test and Pearson's product moment correlation were determined using the statistical package R.

Figure 5.2. UK map, and wider view of South West England indicating the locations of the three field sites, which are enlarged in Figure 5.3.

Figure 5.3. Closer aerial views of the three field sites: Prior's Farm, Quidhampton and Somerset Levels with sampling locations indicated. Land cover types (arable, grassland and woodland) are depicted by the circular symbols.

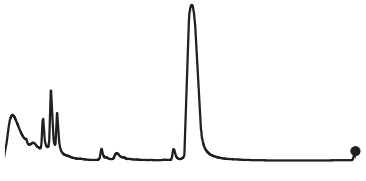
5.5 Results

5.5.1 Ion chromatography

Ion chromatograms of extracts of each control soil type are given in Figure 5.4 with the IP6 peak highlighted in each. The large peak between 10 and 15 min is the EDTA from the extraction solution. Most of the extract material elutes prior to this peak and the IP6 is well separated at ~ 20 min. The matrix material differs in form and abundance between soil types but exploring the nature of this material was outside the scope of this study. The standard addition plots of added IP6 versus peak area (Figure 5.5) gave straight lines with R^2 values of ≥ 0.8781, with most values > 0.95, suggesting that the quantification was not adversely affected by matrix effects.

5.5.2 Soil properties

The soils surveyed and a range of their properties are given in Table 5.1. The soil properties exhibit wide variance within parameters, with pH ranging from 4.6 to 8.3, moisture content between 24 and 79 %, organic content (LOI) from just 7.7 to 83.3 % and total metal concentrations from 3116 to 8228 mg kg^{-1} soil. Some properties have similar values within soil types while others have similar values within land cover, for example, of the soil types, the peat soils displayed the highest organic contents. Among the land covers, the woodland soils had the highest LOI. The peats were also the highest in moisture content, followed by the alluvial soils collected at Quidhampton, then the clay soils from Priors Farm. Alluvial soils had the highest pH, and clay soils the lowest. The peats were not strongly acidic as they were fen peats on a lime-rich bedrock, as reflected in the relatively high calcium concentrations. The alluvial soils exhibited high total metal concentrations, particularly calcium in the arable soil, while the clay soils displayed characteristically high iron and aluminium, and low calcium concentrations.



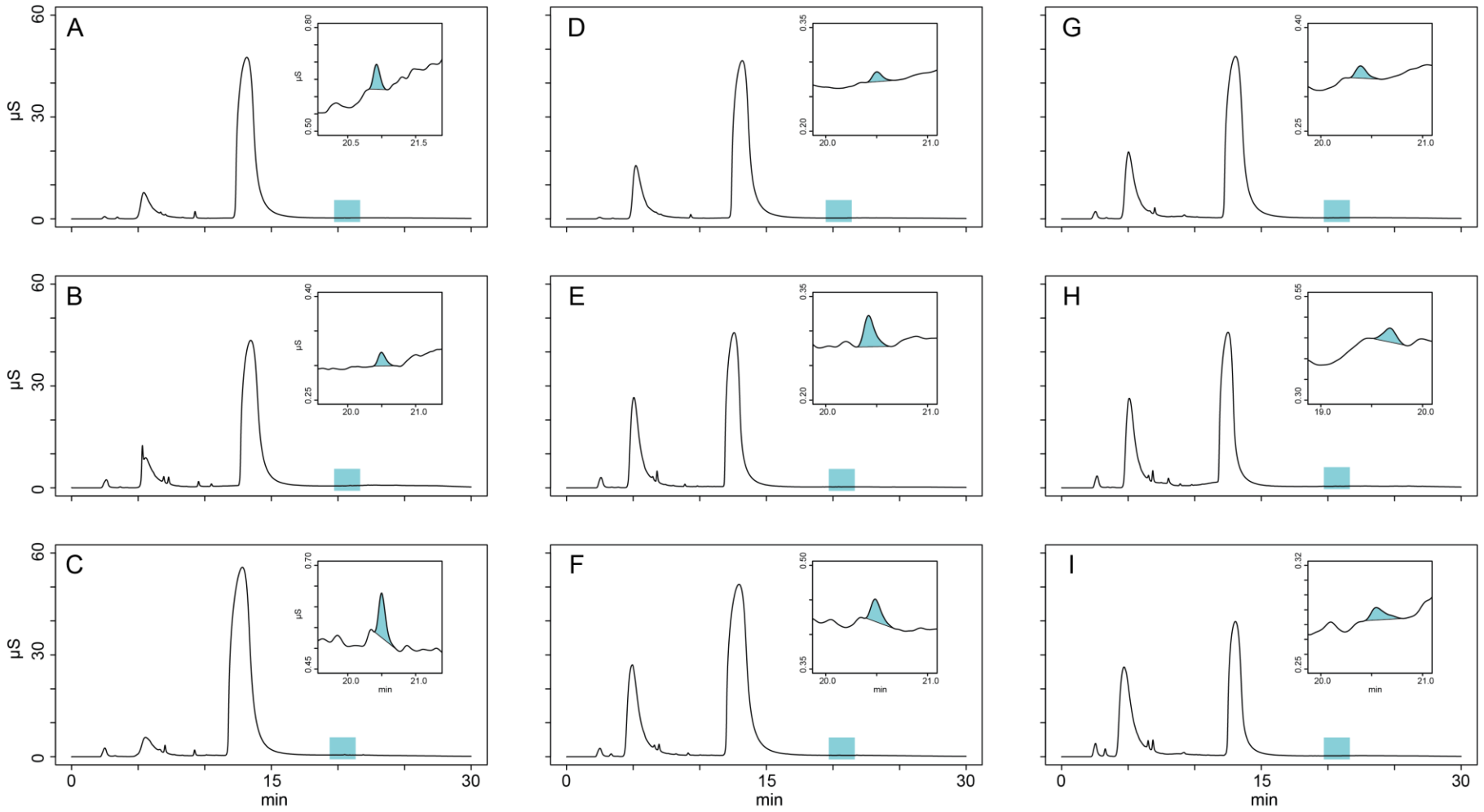


Figure 5.4. Ion chromatograms of each soil type and land cover. (A) Clay, arable soil, PFAR, (B) clay, grassland soil, PFGR, (C) clay, woodland soil, PFWD, (D) alluvial, arable soil, QHAR, (E) alluvial, grassland soil, QHGR, (F) alluvial, woodland soil, QHWD, (G) peat, arable soil, SLAR, (H) peat, grassland soil, SLGR, (I) peat, woodland soil, SLWD. The IP6 peak is highlighted in the enlarged region inset in each chromatogram.

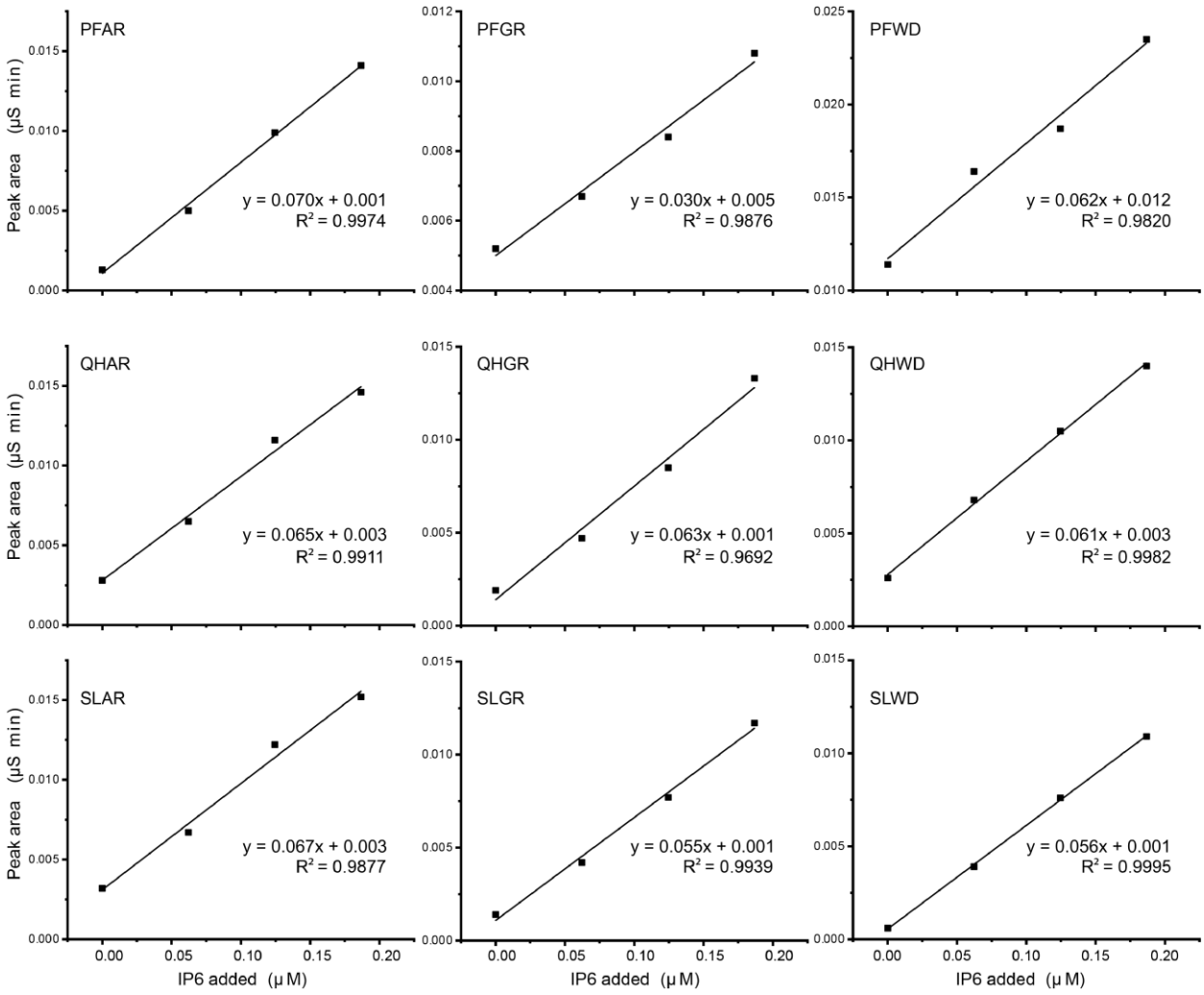


Figure 5.5. Standard addition plots of control soils of each soil type and land cover. Top row are the clay soils, middle are the alluvial, and the bottom row are the peat soils. The left-hand column are the arable soils, the middle are the grassland and right hand column are the woodland soils. R^2 values lie between 0.9692 and 0.9995.

5.5.3 Extraction efficiencies

The average IP6 concentrations for control and spiked soils and the EEIP6 and EETP for each soil are given in Table 5.2. On average, the alluvial soils had a higher EEIP6 than the clay and peat soils. However, the only significant ($p \leq 0.01$) difference between EEIP6 values was found by T-test between clay and peat soils. From these data, there did not appear to be a distinct relationship between EEIP6 and soil type or land cover. The extraction efficiency of NaOH-EDTA for total P (EETP) was poor across all soils, with the highest (29.7 %) being from the clay woodland soil (PFWD).

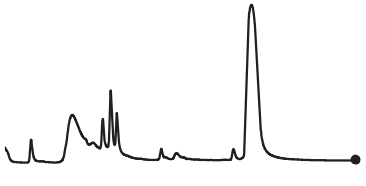


Table 5.1. Soil types, land cover and soil properties. Soil types are clay (Prior's Farm, PF), alluvial (Quidhampton, QH), and peat (Somerset Levels, SL). Land covers are arable (AR), grassland (GR) and woodland (WD).

Sample name	Soil type	Land cover	Moisture content	pH	Organic content LOI	Total P	IP6	NaOH-EDTA extracted P	Al	Ca	Fe	Mg	Mn	Total Metals
			(%)		(%)	(mg kg^{-1})								
PFAR	Clay	Arable	38	7.37	10.7	2006	7.2	385.9	2456	1087	2781	288	22	6633
PFGR	Clay	Grassland	24	6.08	11.9	1332	52.8	293.8	2586	354	2806	227	33	6006
PFWD	Clay	Woodland	36	4.60	20.2	1162	61.2	344.5	2371	212	1766	218	14	4580
QHAR	Alluvial	Arable	66	8.33	7.7	2055	26.2	392.1	1531	4828	1537	260	73	8228
QHGR	Alluvial	Grassland	53	7.02	31.5	1771	3.4	153.1	1988	1435	2525	266	39	6253
QHWD	Alluvial	Woodland	67	6.52	57.9	2298	15.8	308.3	785	1954	1446	139	29	4353
SLAR	Peat	Arable	60	5.28	78.9	1281	13.8	161.4	449	1620	899	208	17	3194
SLGR	Peat	Grassland	73	6.25	69.0	1724	18.8	193.5	1067	1770	1204	267	30	4339
SLWD	Peat	Woodland	79	5.85	83.3	1103	3.8	216.2	154	2187	552	212	12	3116

Table 5.2. Control and spiked soil IP6 concentrations (n = 3) and standard deviation, and determined EEIP6 and EETP

Sample name	Control soil IP6	Spiked soil IP6	EEIP6	EETP
	(mg kg^{-1})		(%)	
PFAR	7.2 ± 2.9	164.9 ± 35.7	69.4	19.2
PFGR	52.8 ± 24	188.9 ± 57.2	66.7	22.1
PFWD	61.2 ± 8.7	181.0 ± 59.4	62.1	29.7
QHAR	26.2 ± 27.3	208.0 ± 62.0	81.0	19.1
QHGR	3.4 ± 2.5	207.9 ± 57.5	88.9	8.6
QHWD	15.8 ± 3.0	152.9 ± 24.9	62.1	13.4
SLAR	13.8 ± 8.8	105.0 ± 32.4	43.0	12.6
SLGR	18.8 ± 16.4	101.15 ± 27.9	40.6	11.2
SLWD	3.8 ± 1.6	93.7 ± 43.1	40.0	19.6

5.5.4 Correlation between EEIP6 and soil properties

The relationships between EEIP6 and total metal concentration, organic content, Fe concentration, pH, IP6 concentration, and EETP are plotted in Figure 5.6 (A – F) and their Pearson's product moment correlation (r) given. EEIP6 correlated positively, and most strongly, with total metal concentration ($r = 0.8472$, $p < 0.01$), and negatively with LOI ($r = -0.8039$, $p < 0.01$). There were positive correlations between EEIP6 and individual metal concentrations (see Figure A1.1 in the Appendix), but these were not found to be statistically significant. The correlation between EEIP6 and Fe had $p \leq 0.05$ ($r = 0.7259$, $p = 0.0268$). However, the correlation with the total metal concentration was still stronger and more significant. While there was a weak positive correlation between EEIP6 and pH ($r = 0.6080$), it was not found to be significant at $p \leq 0.05$. No significant correlation was found for EEIP6 with soil moisture content, total P, or NaOH-EDTA extracted P. No correlation was found between EEIP6 and EETP or control soil IP6 concentration.

The relationships between EETP and soil properties were also investigated, and Pearson's product moment determined. These are shown in Figure 5.6 (G – K). The only significant correlation found at the $p \leq 0.05$ level was between EETP and control soil IP6 concentration ($r = 0.7465$, $p = 0.0209$). There were no correlations between EETP and total metals, LOI, Fe or pH.

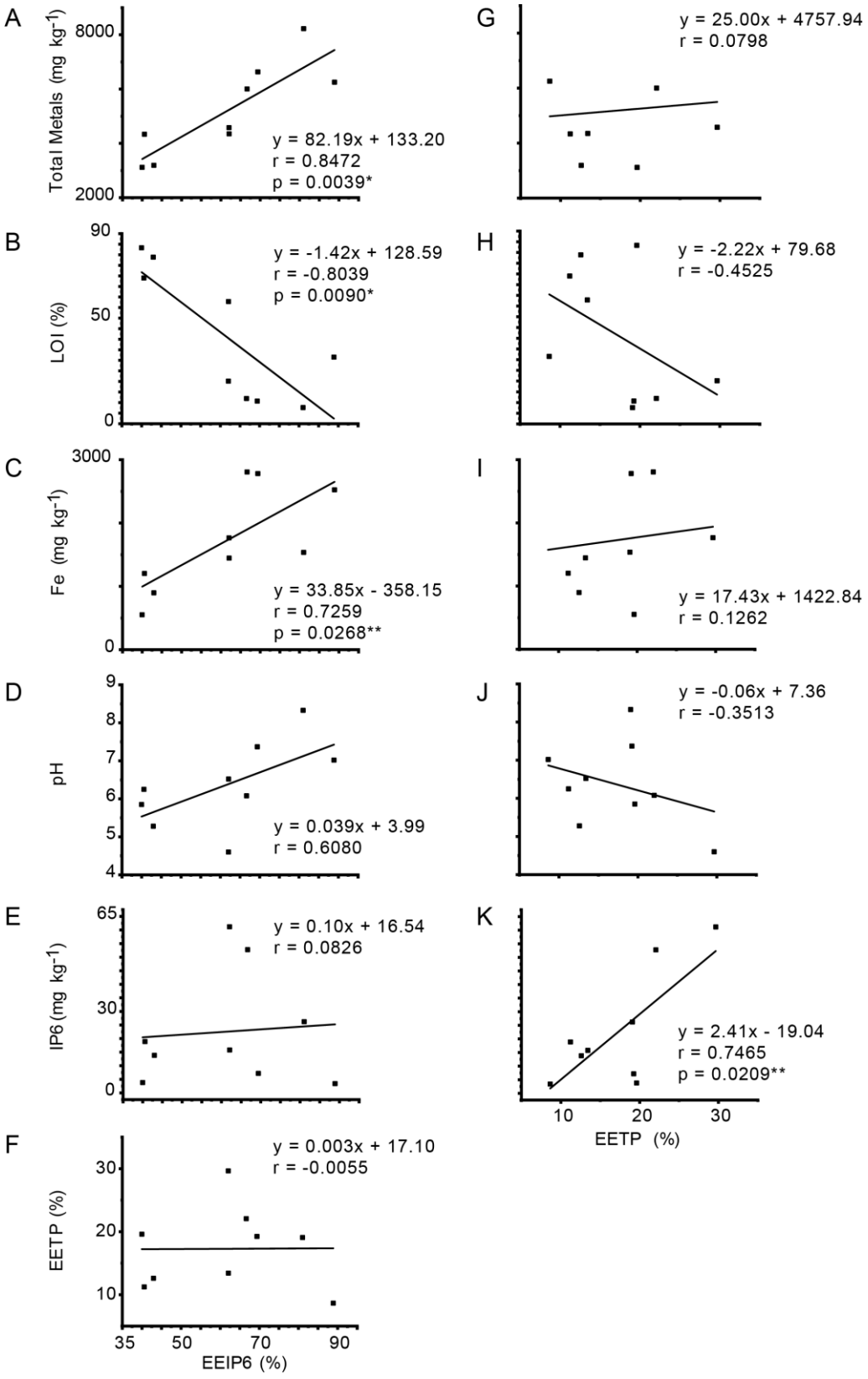


Figure 5.6. Pearson's product moment correlations between EEIP6 (A – F) and Total metals, LOI, Fe, pH, IP6 control soil concentration and EETP. Correlations between EETP (G – K) and Total metals, LOI, Fe, pH, IP6 and control soil concentration. * $p \leq 0.01$, ** $p \leq 0.05$.

5.6 Discussion

The results of this study into the extraction efficiency of the NaOH-EDTA one step extraction method for IP6 reveal some important relationships between the EEIP6 and soil properties. While it may be expected that similar soil types or land cover types will have similar EEIP6, it seems that is not the case, and that more fundamental characteristics of the soils govern the interaction of IP6 with the extraction solution and the soil material. It has been reported that the soil properties that most influence EETP are organic substances, pH and total metal concentration (Giles *et al.*, 2015), hence the further investigation of these properties here.

5.6.1 EEIP6 and soil metal concentrations

The observation of a positive correlation between metal content and EEIP6 was unexpected as it is well-established that IP6 sorbs readily to metals in soils. IP6 has been shown to have a higher Langmuir coefficient of adsorption than PO_4^{3-} for calcite (Celi *et al.,* 2000) and a range of phyllosilicate systems (Celi *et al*., 1999). Yet the strongest correlation observed was that between EEIP6 and total metals. This effect contrasts with Giles *et al.* (2015) who found that *lowest* EETP coincided with high total measured metal concentrations and hypothesised that the EDTA in the extraction solution was saturated with metals, leading to lower P recovery. Indeed, total metal concentrations determined in their study were up to 10 times higher than those found here. Where EDTA is not saturated with metals, it appears to be a very efficient extractant for compounds bound to metal ions, such as IP6. The relationship between EEIP6 and metal concentrations appears most influenced by Fe which also had a positive correlation ($r = 0.7259$, $p = 0.0268$). Agbenin & Tiessen (1994) reported that aluminium oxide has a greater effect on P adsorption than Fe oxides and this effect appears to be borne out here.

5.6.2 EEIP6 and organic matter content

The inverse relationship whereby high EEIP6 correlated with low organic matter content (Figure 5.6 B) further confirms the correlation with total metals as high mineral soils inevitably have low organic content and vice versa. IP6 is thought to bind to complex organic material in soils *via* Fe or Al bridges (Gerke, 2015a). Therefore, it was surprising to see the lowest apparent retention in soils with high total metal concentrations, and in particular with increasing Fe concentration (Figure 5.6 C). The correlation between EEIP6 and Al was also positive with $r = 0.6592$ (see plot in Appendix), and just above the $p \leq 0.05$ threshold.

The correlation between high pH and high extraction efficiency ($r = 0.6080$) was expected to be stronger, especially given that the extraction itself is basic. In some studies, it has been found that acidic conditions lead to the accumulation of IP6 (Turner & Blackwell, 2013, Anderson & Arlidge, 1962, Caldwell & Black 1958. It is surprising then that the pH of soils did not have a stronger influence on EEIP6 like that of metals and LOI.

5.6.3 EETP and soil characteristics

It is striking that the relationships determined for extraction efficiency of IP6 from soils and the soil characteristics determined here are not found for EETP. The only significant correlation between EETP and other soil parameters was between that and control soil IP6 concentration (Figure 5.6 K) at the $p \leq 0.05$ significance level. Unlike those found for EEIP6, no relationships were found between EETP and total metal concentration, LOI, Fe, or pH.

No correlation was found between EEIP6 and control soil IP6 concentration. This indicates that the factors governing the observed sorption and accumulation of IP6 in soils (Anderson *et al.,* 1974; Celi & Barberis, 2006) differ from those governing the release of IP6 to the extraction solution. The lack of correlation between EEIP6 and soil total P, and EEIP6 and EETP also indicate the mechanisms involved in the accumulation and retention of bulk P differ from those

governing soil-IP6 and IP6-extraction solution interactions. Of course, the soil P profile consists of a myriad of P compounds with a wide spectrum of physico-chemical properties which will have a broad range of interactions with soil matrices.

5.6.4 NaOH-EDTA extraction of P-containing compounds from the soil matrix

It appears that the basic EDTA extraction method employed here is well suited to the release of IP6 from soils to solution in preference to the majority of other P-containing compounds. EDTA competes with the phosphate groups of IP6 effectively for metal ions, easily displacing the compound. The hydroxide ions in the extraction solution must also compete with phosphate groups for binding sites on soil organic material. However, these binding sites will vary greatly in physical and chemical form and extraction efficiency will not be uniform across all conditions. For example, steric hindrance by bulky organic molecules may explain the lower effectiveness of OH^- ions for release of IP6 from the matrix. Steric hindrance may also negatively affect the efficiency of EDTA for the release of IP6 bound to organic compounds *via* cationic bridges.

A potential implication of this study is that it should be taken into account that NaOH-EDTA extractions of soil and other matrices preferentially releases IP6 to solution versus other P compounds. It appears that the extraction is particularly efficient in this respect, extracting 83.4 % of IP6 in the clay arable soil, at the same time as extracting 19.2 % of total P. It is worth considering that quantification results from extractions of soils using this method may therefore over-estimate IP6 concentration relative to total P. In particular, soils with high total metal concentrations and low organic content can be expected to have high extraction efficiency for IP6, but not necessarily for total P extracted with NaOH-EDTA.

5.6.5 Implications for soil-IP6 interactions

Whether these trends reflect the strength of binding of IP6, or even P, to soil cannot be determined from this study alone. There have been plenty of studies of the *sorption* of IP6 to soils (Anderson & Arlidge, 1962; McKercher & Anderson, 1989; Celi *et al*., 1999, 2000), but the strength of sorption does not necessarily relate to magnitude of *retention* (Williams *et al.,* 1958). If the data here can be extrapolated and mechanisms of release of IP6 from soil are reflected in these results, then the accumulation of IP6 in soils may not be assumed to be related directly to the sorption properties of the soil, as is often inferred (Stewart & Tiessen, 1987; Gerke, 2015a; Celi *et al*., 1999). Similarly, the use of extraction solutions as a proxy to infer bioavailability of P pools is a frequently disputed topic (Liu *et al*., 2015) and this study adds to that debate, as the investigation of the availability of individual compounds to extraction solutions reveals variation in response to that of the total P fraction under the same conditions.

The factors governing extraction of IP6 from soils taken into account in this study are all presumed to be physico-chemical. The two-hour incubation period and use of air-dried, 2 mm sieved soil were considered insufficient conditions for biological activity to appreciably alter the results. The effect of biological factors, such as fungal or bacterial activity, plant root exudates, enzymes and macrofauna have not been considered here (Ryan *et al.,* 2001; Gerke, 2015b). Further study of retention mechanisms under field physical and biological conditions would need to be undertaken to understand their effects on EEIP6. A wider study with more soil types and particularly those with higher metal concentrations is recommended.

These data demonstrate that the effect of NaOH-EDTA extraction on soil IP6 diverges from that of soil total P, and that soil type or land cover are not the best indicators of this behaviour. Soil properties are more important indicators of the efficiency of the extraction and differ for the individual compound and the bulk fraction. The use of the IC standard addition

quantification method has enabled a detailed study into the molecular level mechanisms of IP6 extraction from soils. The advantage of this method is its rapidity, enabling large numbers of extracts to be analysed in a short timeframe for a comprehensive survey. It is only by looking in such detail at the molecular level that the impact of P compounds on the environment can be fully elucidated.

While these data reveal an unprecedented insight into molecular interactions, the results of this study should be interpreted with caution. Extraction efficiencies of IP6 and TP are not quantitative. Recovery of IP6 ranges from 40 to 90 %, and recovery of TP is particularly low, with highest recovery at 30 %. Where EEIP6 is high and EETP is low, there is clearly a bias in the extraction in favour of IP6. Low EE for both IP6 and TP indicate that further study, particularly into the extraction conditions, is necessary to gain full insight into the mechanisms of binding and extraction of P compounds in these and other soils.

5.7 Conclusions

The method of IP6 quantification using standard addition in IC has enabled the detailed exploration of EEIP6 of a range of soils and the relationship of soil properties to EEIP6. The main findings of these investigations are that:

1. It was found that neither soil type nor land cover was a good predictor of extraction efficiency and that more fundamental soil characteristics were involved in soil-IP6 and IP6-extraction solution interactions.
2. A significant positive correlation was found between EEIP6 and total metal concentration. A significant ($p \leq 0.01$) negative correlation was found between EEIP6 and LOI. A less significant ($p \leq 0.05$) correlation was found between EEIP6 and soil Fe concentration.

3. The data indicate that soils with high total metal concentration, and low organic content will have the highest EEIP6 using NaOH-EDTA extraction. This was unexpected given the reported propensity of IP6 to sorb to soil mineral surfaces.
4. The same relationships were not observed for total P extraction efficiency. Therefore, there is a divergence in the behaviour of IP6 from the bulk P pool in soils.
5. These results have implications in relation to the reliability of quantification of IP6 from NaOH-EDTA soil extracts. It should be taken into account that this extraction procedure is selective for IP6 over total P and that IP6 quantification may be overestimated for mineral soils or underestimated for organic soils using this extraction method.

The bulk determination of total P as a characteristic of soils gives a broad indication of the impact of P on environmental systems, but clearly misses the detail of the behaviour of individual compounds. IP6 is an important soil organic P compound and its interactions with soil and the NaOH-EDTA extraction solution diverge considerably from those of the bulk P fraction and varies with intersecting soil properties, even within soil type. This study shows the importance of molecular level determination of mechanisms to further our understanding of the P biogeochemical cycle. This quantification method has proved valuable to in-depth study of IP6 in soils.

Chapter 6. Determination of fate of IP6 from poultry litter in soil in incubation and leaching experiments

6.1 Abstract

IP6 in manure is a potential source of P to plants in soils and, through leaching from soil, a potential contributor to eutrophication. The degradation of IP6 has previously been studied after application of a commercial IP6 solution to soils and analysis with ^{31}P NMR. The transport of IP6 from poultry litter has also been studied through NMR and it has been found to be mobile in leaching events in the weeks post application. However, the scope of these studies was necessarily limited due to the time-consuming nature of ^{31}P NMR analyses. Here, the quantification of IP6 using IC has been applied in a high-resolution study of the fate of IP6 in soils from both a commercial solution and a natural source in the form of poultry litter. A preliminary transport study was also conducted to determine if incubation time effected the mobility of the compound during leaching of a soil column. The results demonstrate dynamic behaviour of IP6 in soils with increased concentrations in the first two weeks post-application, followed by degradation. Incubation of soil columns prior to leaching, for two weeks and one day, confirmed this behaviour. Visible fungus and soil core pH measurements point to microbial-mediated release of the compound from the poultry litter. These studies highlight the need for in-depth analysis of IP6 behaviour under natural conditions. The release and decomposition of IP6 from poultry litter has implications for soil IP6 concentrations, but the compound does not appear to be more mobile after incubation.

6.2 Introduction

As one of the major organic P compounds in soils, IP6 has the potential to be an important source of P, particularly under low phosphate conditions (Turner, 2007). Plants are incapable of acquiring the compound directly but exude organic acids from their roots to interrupt binding

of IP6 to soil minerals (Ryan *et al.,* 2001). Microbial activity, particularly extracellular phytase enzymes, hydrolyse IP6 releasing phosphate for uptake by plants from the soil solution (Sims *et al.,* 2005). The potential for degradation of IP6 in soils is not well understood as it has long been considered to be tightly bound to soil particles and therefore physically protected from enzymatic hydrolysis (Turner *et al.,* 2003c) and resistant to mobilisation in the soil solution (Leytem *et al.,* 2002). A major input of IP6 to soils is from the application of manure fertilisers, particularly those from pigs and poultry who lack the ability to digest the compound (Humer *et al*., 2015). The IP6 content of these manures can be quite high as seen in Chapter 4 of this thesis and in other studies (Li *et al.,* 2014; Turner, 2004). Understanding the fate of the compound applied in manures and therefore its potential to contribute to soil fertility as well as its mobility is essential to optimise the use of organic fertilisers and to prevent unintended pollution of waterways from runoff.

Previous studies have used ^{31}P NMR to investigate the degradation of IP6 in soils (Doolette *et al*., 2010, 2011a), and its leaching from poultry litter applied to soil columns (Giles *et al.,* 2011). Due to the lengthy and intensive nature of sample preparation and analysis using NMR, the number of timepoints and/or replicates in these studies has been limited. Doolette *et al.* (2010, 2011a) investigated the degradation of IP6 from a commercial IP6 standard solution over a 13-week incubation in a calcareous soil. In the 2010 study, the IP6 was added to soil at a rate of 58 mg kg^{-1} with, and without, straw. Two replicates for each treatment were extracted at Weeks 0, 1, 4, 7 and 13. A first-order decay rate was fitted and a half-life of IP6 in the soil of 4 -5 weeks was determined (Figure 6.1 A, B).

For the 2011 study, a range of IP6 concentrations, again in the form of a commercial IP6 solution, were applied to the soil and the same timepoints and number of replicates analysed. The first-order decay rate was fitted and the half-life of IP6 was found to be between 4 and 8

weeks (Figure 6.1 C), although there was no relationship between the magnitude of IP6 applied and length of half-life. The decrease in concentration of IP6 coincided with an increase in PO_4^{3-} extracted from the soil, indicating that the IP6 was being broken down into PO_4^{3-} by microbial activity.

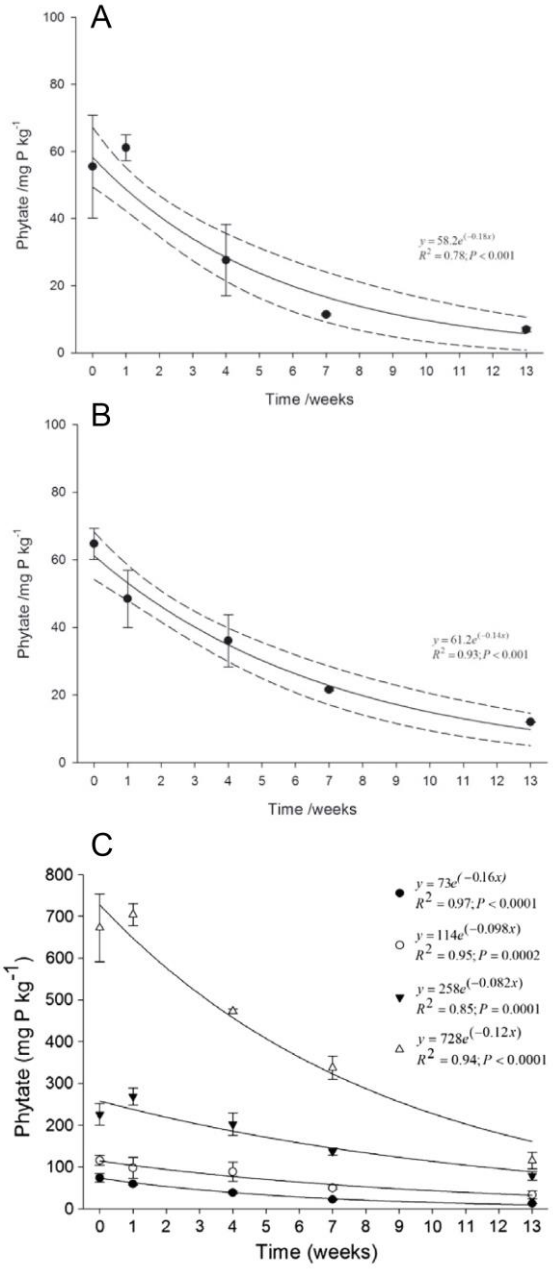


Figure 6.1. The first-order decay rate model of commercial IP6 solution added at a rate of 58 mg kg^{-1} to soil (A) and to soil and wheat straw (B). The experiment was repeated, and data shown in (C) are for treatments at ● 75 mg kg^{-1}, ○150 mg kg^{-1}, ▼ 290 mg kg^{-1} and △ 730 mg kg^{-1}. Adapted from Doolette *et al.* (2010, 2011a).

These studies contradict the supposition that IP6 is strongly bound to soils, particularly calcareous soils, and is thus immobilised and inaccessible to micro-organisms for utilisation. They demonstrate that IP6 is in flux in soils, and soil microorganisms have strategies to overcome soil-IP6 interactions. While the decay curve fits the data, very few timepoints were sampled and only two replicates analysed. Whether these results reflect the behaviour of IP6 when applied in the form of an organic manure such as poultry litter, as opposed to a commercial IP6 solution, was not addressed. Like soils, manures are complex mixtures of organic and inorganic natural matter in various stages of degradation. Their composition and microbial community differ greatly from those of soil and therefore IP6 binding, degradation and mobilisation processes are likely to be of a different nature to those of commercial IP6 in soil alone.

Giles *et al.* (2015) used ^{31}P NMR to study the transport and transformation of P species, including IP6, in a saturated soil amended with poultry manure after leaching. The three replicate soil columns were leached every week for 10 weeks and samples were taken from one of the columns at the 0 – 5 cm and 10 – 15 cm depths for extraction and analysis by NMR on Weeks 2, 4, 6, 8 and 10. Poultry manure was added on Week 1 and each weekly leaching event was with one pore volume of water. The results indicated that IP6 was transported to the lower depth by Week 4 (three weeks after manure application). Week 4 had the highest concentration of IP6 for both depths in the soil, followed by a sharp decline in the 0 – 5 cm depth to Week 6, with a more moderate decline to Week 8 in the 10 – 15 cm depth. The extracts for ^{31}P NMR derived from a single soil column, means there were no replicates for this experiment. As the experiment combines transport with transformation over time, it is difficult to differentiate between the processes in this study.

While these degradation and transport studies give some insight to the behaviour of IP6 in soils, the use of ^{31}P NMR limits the number of sampling points and replicates achievable. Taking advantage of the rapidity and sensitivity of the newly developed IC method of quantification of IP6 from soil extracts, the study of IP6 in soil can be achieved at a higher resolution. Comparing the degradation of IP6 from a natural source such as poultry litter versus IP6 from a commercial solution will indicate the relevance of the Doolette *et al.* (2010, 2011a) studies to field conditions. In this way we can gain more in-depth information about the cycling of IP6 in soils from poultry litter under representative conditions.

6.3 Aims

The aims of this chapter were:

1. To replicate the studies of Doolette *et al*. (2010, 2011a) using the IC standard addition method of quantification to obtain a higher resolution of sampling with more timepoints and replicates.
2. To determine if there was a difference in behaviour of IP6 in soil from a natural source – poultry litter – and to compare that with IP6 added to the soil in a standard solution.
3. To use the results of this experiment to inform the design of the subsequent experiment whereby the mobility of IP6 from poultry litter after a single leaching event was investigated and the effect of duration of pre-incubation was determined.
4. To sample IP6 at three depths (0 – 5 cm, 5 – 10 cm, 10 – 20 cm), as opposed to the two used in the Giles *et al*. (2015) study.

6.4 Methods

The soil used for these experiments was a BS3882:2015 certified topsoil purchased from Raycox Turf Ltd. (Bristol) and was accompanied by soil analysis results and a declaration of

compliance. The soil was described as slightly calcareous sandy loam with a weakly developed, fine to medium granular structure. It was chosen for these studies as its properties were well-defined, it was homogenised, screened to 20 mm, and available in large quantities. The aim of choosing this soil was to minimise variation between samples, and to facilitate future comparable studies, if required, due to the quantity available.

The poultry manure was collected fresh from the Langford Veterinary School at the University of Bristol. The birds were Columbian black tail commercial layers that were fed on a layer's mash diet, composed of wheat and soya meal, with a daily scatter feed of corn. They were kept at 10 birds to a room.

The incubation was set up with 24 replicates for each treatment (control soil – no treatment (CTRL), poultry litter amended soil (PL), IP6 solution amended soil (PS)) as described in Chapter 2, Section 2.8 and the soil lysimeters as described in Section 2.9. The soil core samples were extracted using the NaOH-EDTA extraction method in Section 2.3 and IP6 was quantified in the extracts using IC with standard addition quantification as in Section 2.10.1. Soil pH of the soil cores at each depth was determined using the method outlined in Section 2.10.8.

T-tests were performed using statistical package R.

6.5 Results

The properties of the topsoil and the poultry litter used in the experiment are shown in Table 6.1. Soil moisture content, water holding capacity and IP6 concentration were determined in the laboratory, as were the moisture content, IP6 concentration, pH and organic content of the poultry litter.

Table 6.1. Details of the BS3882:2015 certified topsoil. Properties were provided by the soil supplier except where * indicates parameters determined in the laboratory.

Property		Soil	Poultry litter
Clay	%	17	
Silt		28	
Sand		55	
Moisture content		18.4*	51*
Water holding capacity	ml kg^{-1}	638*	
IP6 concentration	mg kg^{-1}	5.78*	4,850*
pH		7.8	6.6*
Organic matter (LOI)	%	5.9	86*
C:N ratio		12	
Extractable P	mg L^{-1}	68	

6.5.1 Incubation experiment

The mean IP6 concentration ± one standard deviation for each timepoint for the three treatments is shown in Figure 6.2. Enlarged sections highlight the change in concentration in the first five weeks of the incubation for the PL and PS treatments. For the majority of extracts across all timepoints in the CTRL treatment, the concentration of IP6 is ca. 5 mg kg^{-1} ± 2 mg kg^{-1}. One extract at Week 2 had an elevated concentration of IP6 (43 mg kg^{-1}) relative to all other CTRL extracts across the experiment. This incubation tube contained a germinated seedling, and it is thought that the seedling was incorporated into the sample and contributed the large excess of IP6.

In the results for the PL treatment a large initial increase in the average concentration of IP6 in the soils is observed over the first two weeks, followed by a decline from Week 2 to 11. There is a large variance in the data, particularly for Weeks 1 and 2, however, the IP6 concentration for two out of three extracts for each of Weeks 1, 2, 3 and 5 are above the average concentration in the initial Week 0 extracts. The average IP6 concentration does not fall below the initial Week 0 average concentration until Week 7. By week 11, the average concentration of the three replicates has depleted to 12.65 mg kg^{-1}. Fungus was observed frequently on the surface

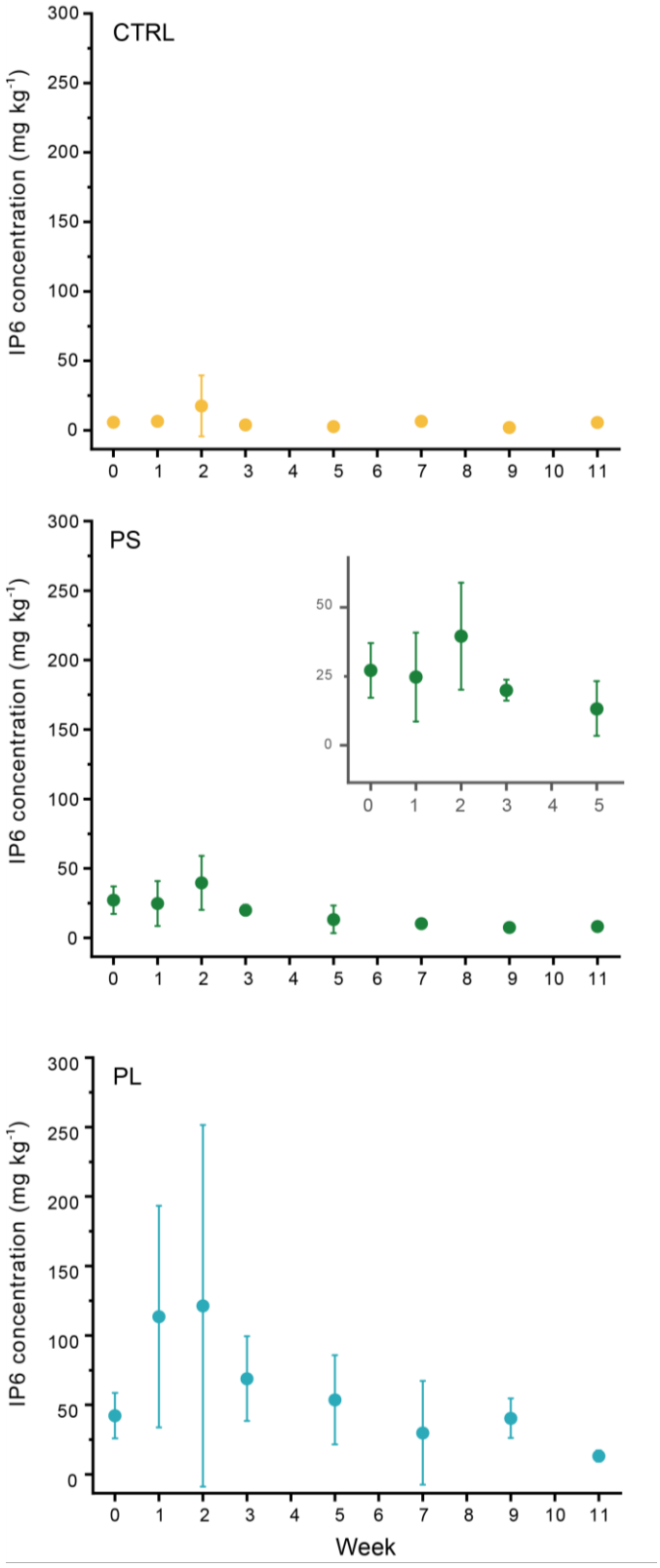


Figure 6.2. Mean concentration of IP6 ± one standard deviation for all treatments at each timepoint: control (CTRL), poultry litter (PL), and phytate solution (PS). Inset highlights the dynamic changes in soil IP6 concentration for the first five weeks for the PS treatment.

of the PL soils over the course of the experiment, and only occasionally on the CTRL and PS soils.

The results for the PS treatment again show variance in the IP6 concentrations for each week. It appears that there is also a slight increase in the average IP6 concentration from Week 0 to Week 2, although the trend is not clear. This is followed by a decline from Week 2 to 11.

6.5.2 Transport experiment

The concentrations of IP6 at the three soil depths in the three treatments (CTRL, PL 1, and PL 14) are shown in Figure 6.3. It is clear a large difference in the IP6 concentrations exists between the CTRL, PL 1 and PL 14 treatment at 0 – 5 cm. The difference is not as marked between the treatments at the 5 – 10 cm depth, and there are no differences in concentrations at the 10 – 20 cm depth. From these data it can be seen that the PL 14 treatment has the highest concentrations of IP6 in the upper two sections of the soil core, and that for both the PL 1 and PL 14 treatments there is a decrease in concentration down the core.

Fungus was observed on the surface of the PL 14 soil columns from 7 days onward indicating favourable conditions for microbial growth. It can be seen clearly in the photograph in Figure 6.4 and its influence can be seen in the pH results.

The average pH of the soil cores at each depth is shown in Figure 6.5. The pH of the CTRL soil is highest at the top (pH 8.44) and follows a linear downward trend to pH 8.02 at the lowest depth. The PL 1 pH results follow a similar, but not linear, trend. The pH at the 0 – 5 cm level is lower (pH 8.00) than that of the CTRL soil core at 0 – 5 cm. The PL 14 soil has the lowest pH at all levels, but particularly the 0 – 5 cm level (pH 7.54). In contrast to the CTRL and PL 1 results, pH is higher in the 5 – 10 cm section than the upper section and falls again at the 10

– 20 cm depth. The soil core pH values for PL 1 and PL 14 are almost identical at the 5 – 10 cm and 10 – 20 cm depths.

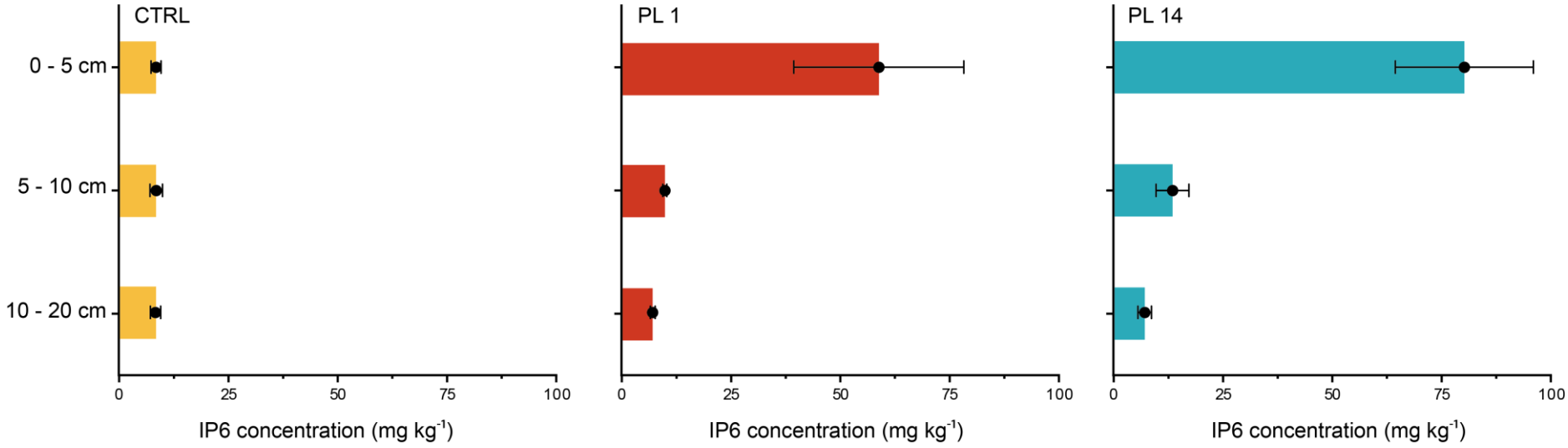


Figure 6.3. Mean (n = 3) IP6 concentrations ± one standard deviation at each depth of the soil column for the three treatments: control soil (CTRL), soil with poultry litter post 1-day incubation (PL 1), soil with poultry litter post 14-day incubation (PL 14). There is no change in CTRL soil IP6 concentrations, but a clear change with depth for treatments PL 1 and PL 14. Significant differences were found between the means at each depth for both these treatments.

Figure 6.4. A photograph of the surface of a PL 14 soil column on Week 2. The white mycelium of the growing fungus can clearly be seen in the upper right quadrant of the surface, circled in blue.

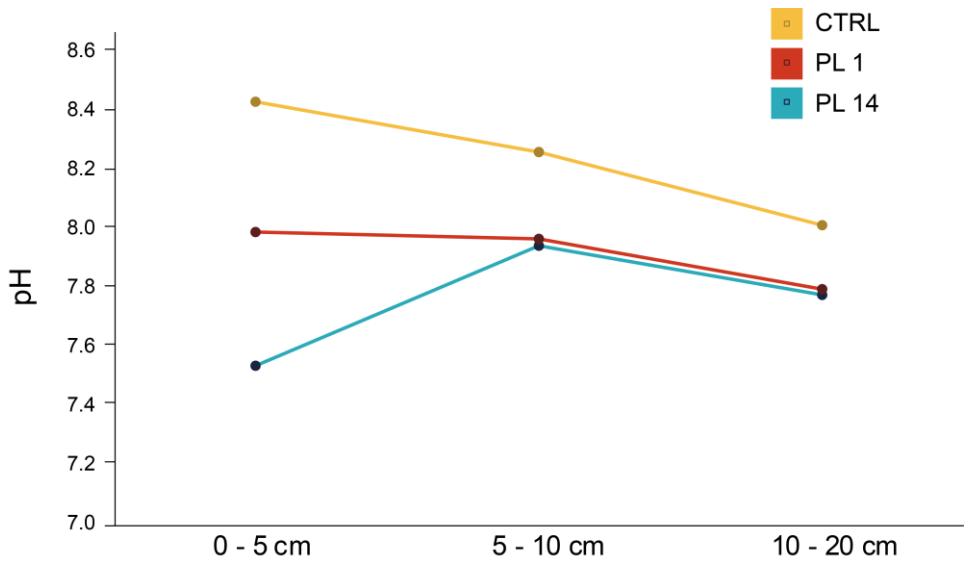


Figure 6.5. Average pH of the soil cores at each depth.

Finally, Table 6.2 shows the average (n = 3) percentage of the total IP6 for each treatment at each depth for the PL 14 and PL 1 soil columns. Despite different total amounts of IP6 in each treatment the proportion of IP6 at each depth is almost identical.

Table 6.2. Percentage of total IP6 for treatments PL 14 and PL 1 at each depth. Calculated from the average IP6 concentrations.

	PL 1 **%**	**PL 14** **%**
0 – 5 cm	88.4	87.6
5 – 10 cm	8.1	9.9
10 – 20 cm	3.5	2.5

6.6 Discussion

6.6.1 IP6 from poultry litter

The data from this experiment shows an unexpected dynamic behaviour of IP6 from poultry litter when incubated in soil. Rather than depletion in concentration from Week 0 over the time course as seen in Doolette *et al.* (2010, 2011a), there appears to be an initial large increase in the concentration of IP6 in the PL treatment. This increase is far above the 82.5 mg kg^{-1} concentration expected for the soils based on the quantification of IP6 in the poultry litter and the fertilisation rate. The data suggest that IP6 in poultry litter is in a recalcitrant form and is inaccessible to the NaOH-EDTA extraction. The implication then is that some process in either the soil or the litter itself, facilitates the release of IP6 from this recalcitrant form in the early stages of the incubation. This processing makes the compound available to the soil matrix and therefore extraction and quantification. After release, IP6 is then consumed or degraded by, most likely biological, but also perhaps physical processes in the soil.

This phenomenon can potentially be explained by considering the form of IP6 in the poultry litter and the diet from which it originates. The poultry diet contains a high grain content. In

this case the layer's mash had high proportions of wheat and soybean meal. IP6 P can constitute up to 60 - 82 % of total P in the grains (Ravindran *et al.*, 1994). IP6 has been found to have antinutritional properties for monogastric animals, such as poultry and pigs, reducing intestinal absorption of minerals, proteins and starch and phytase is often added to monogastric animals' diets to counteract this effect (Woyengo & Nyachoti, 2013). It is found in protein bodies in grains – in the aleurone layer of wheat grains and the endosperm of legume seeds (Gupta *et al.,* 2015). The combination of protein and IP6 in grains may be the cause of the recalcitrance of IP6 to the extraction procedure.

6.6.2 IP6 complexes in poultry nutrition

The interaction of IP6 with seed constituents in grain-based diets has been the subject of a lot of study to better understand its role in the diet. For example, the compound has been found to form insoluble precipitates with Ca (Grynspan & Cheryan, 1989) limiting calcium absorption. Hill & Tyler (1954) found IP6 associated with wheat gluten to be insoluble at pH 2 – 5 and it has been found to form insoluble precipitates with soybean proteins (Rham & Jost, 1979). The importance of insoluble protein-IP6 complexes in animal nutrition has become more apparent and over the last few decades their interaction with dietary minerals and gut pH have become the focus of nutrition studies. IP6 binds with proteins from soya (Rham & Jost, 1979), wheat (Hill & Tyler, 1954), rapeseed (Gillberg & Törnell, 1976) and peanut (Fontaine *et al*., 1946). The mechanisms of the formation of these complexes are varied and several alternatives have been recognised. The first proposed complex, and most important for nutrition, is a binary protein-IP6 complex whereby it's thought that IP6 forms "salt-like" bonds with basic amino acid residues (arginine, histidine and lysine) of proteins at pH values below their isoelectric points (Cheryan & Rackis, 1980). This causes the protein and IP6 to form an aggregate,

providing physical protection from digestion. Ca^{2+} ions can promote the disruption of these protein-IP6 complexes as observed by Okubo *et al*. (1976).

An alternative structure is the formation of ternary protein-IP6 complexes where the IP6 and protein are linked *via* a cationic bridge at pH values above the isoelectric point of the protein. For example, Nosworthy & Caldwell (1988) observed precipitation of soya glycin with Zn and IP6 at pH 6.2. These ternary complexes are not as recalcitrant as binary complexes and so are less disruptive to protein digestion.

A third proposed mechanism is where IP6 acts as a "Hofmeister anion" given that HPO_4^{2-} is recognised in the Hofmeister anion series (Baldwin, 1996). It is hypothesised that the hydration shell of the anion disrupts the interaction of the protein with surrounding water molecules and decreases the solubility of the protein but without direct interaction with IP6 itself (Cowieson & Cowieson, 2011)

The form of binding between IP6 and starch is not understood, but starch but IP6 has been found in close association with sorghum starch. Studies have shown that IP6 coincides with reduced digestibility of starch (Selle *et al.*, 2012, Rooney & Pflugfelder, 1986).

6.6.3 Recalcitrance of protein-IP6 complexes to extraction

The propensity of IP6 to form insoluble precipitates with macronutrients is clearly influenced by multiple factors of diet and gut pH. However, it is a phenomenon that occurs regularly with proteins from various grain sources. It is therefore hypothesised that the elevated IP6 concentrations found here in the early stages of incubation are due to the reduced digestibility of these dietary components, particularly protein, complexed with IP6 inevitably passing through the poultry gut, arriving unaltered in the poultry faeces. Protected by the insoluble macro-molecular complexes, IP6 is then undetectable by the extraction procedure. As the

poultry litter is broken down by soil microbes over the course of the incubation, the protein-IP6 complexes are hydrolysed by microbial enzymes, releasing IP6 and making it available for extraction.

These data and the previous research into protein-IP6 complexes from grains indicate that the concentration of IP6 determined in the initial extraction of the poultry litter greatly underestimates the actual amount present. The IP6 cannot be detected until the grain protein-IP6 complexes are broken down by soil microbes. Yet Leytem *et al.* (2008) reported similar concentrations of IP6 extracted from poultry litter using NaOH-EDTA and 0.5 M HCl extractions. IP6 in the NaOH-EDTA extracts were determined using ^{31}P NMR and IP6 in the HCl extracts was determined using IC. Rippner *et al.* (2014) found extraction efficiencies of 80 – 100 % when IP6 was spiked into broiler litter prior to extraction. However, it is unlikely that the IP6 spike will take the form of the complex protein-IP6 structures that occur in poultry faeces on simple mixing in solution, so this high recovery may not be a realistic estimate. As we saw in Chapter 5, recovery of IP6 using NaOH-EDTA is lower for soils with high organic content. The 86 % LOI of the poultry litter would be in accordance with that finding, and potentially the NaOH-EDTA extraction conditions are even less efficient in contention with protein-IP6 complexes. An enzymatic treatment that targets proteins for hydrolysis may be necessary for complete degradation of poultry litter and determination of IP6 or a well-controlled incubation study whereby poultry litter is degraded microbially with frequent sampling, may offer more insight to the processes involved.

The high-resolution sampling technique employed here (three replicates, weekly sampling for the first four samples) facilitated by the use of IC determination has revealed an unexpected, dynamic behaviour of IP6 from poultry litter. This resolution of sampling, if not more frequent with more replicates, should be used in any future investigations.

6.6.4 Behaviour of IP6 from commercial solution

A similar, but less pronounced trend may be seen in the concentration of IP6 in the PS treatment. There appears to be a slight increase in the concentration of IP6 from Week 0 to 2. This is clearly unrelated to protein-IP6 complexation, and whether this is due to variance in the data, rather than the physical behaviour of the compound is not clear. Perhaps some physical stabilisation of IP6 in soil occurs when the compound is applied in solution, and biological activity is required, for example release of oxalate by fungi, to reverse the stabilisation. Revisiting the data in Doolette *et al.* (2010, 2011a) a similar phenomenon appeared to occur in week 1 of some of the experiments (Figure 6.1, A). In the 58 mg kg^{-1} (2010) treatment the average concentration of IP6 is elevated relative to Week 0. In the 2011 study again, two of the four treatments have Week 1 averages higher than Week 0, followed by depletion through the next 3 timepoints (Figure 6.1, C). Whether this is a real phenomenon or coincidentally similar variance in the data is not clear. The Doolette studies have just two replicates per treatment, five timepoints, and no data covering the period between Week 1 and Week 4. This makes it difficult to determine if there is any deviation from the fitted degradation curve in the early part of the experiment. Further study is required to identify if there is a trend, and to then elucidate the factors causing this effect.

There is no change in IP6 concentration in the CTRL soils. Aside from the anomaly in Week 2, the concentration of the compound in the soil in the absence of any treatment stays in an equilibrium. The IP6 contribution from the seedling is substantial, resulting in an IP6 concentration 8 times that of the baseline soil concentration. Germinated seedlings were observed in just under a quarter of replicates across all treatments, but they did not develop due to being incubated in the dark. As far as could be seen, no other extracts incorporated seedlings.

6.6.5 Transport study

The dynamic behaviour of IP6 observed in the incubation experiment revealed a need for more detailed study of this compound when applied in organic manures, such as poultry litter. A preliminary transport experiment was planned to further study the mobility of IP6 from poultry litter and the results of the incubation experiment informed the design.

Comparing the average IP6 concentrations at the top of the soil cores for PL 1 and PL 14, an elevated concentration of IP6 is again observed after two weeks of incubation versus one day. A T-test indicates that the difference is not significant at the 0.05 level, likely due to the high variance seen in the data. There were, however, significant differences between the concentrations of IP6 between 0 – 5 and 5 – 10 cm depths, and between 5 – 10 cm and 10 – 20 cm depths for both the PL 1 and PL 14 treatments. This indicates that at equilibrium flow (whereby volume of leachate collected after each rainfall event was constant), the IP6 had begun to move down the soil column, although the majority of the compound remained at the surface. The IP6 does not appear to have reached the bottom of the column in this timeframe as the concentrations at 10 – 20 cm amongst all of the columns were comparable to the control soil IP6 concentration.

The fungus observed at the top of the PL 14 soil columns and the pH of the soil cores at the 0 – 5 cm level indicate an active microbial community after incubation. Fungi are known consumers of IP6 with over 200 fungal isolates found to produce extra-cellular phytase (Gupta *et al.,* 2015). Mycorrhizal fungi exude organic diacids such as oxalic and citric acid along with phytase to aid the release of phosphate from IP6 (Sims *et al.,* 2005). The poultry litter was not sufficiently acidic relative to the soil and at the applied rate to have affected the soil pH to the extent seen in Figure 6.5. The coincidence of the appearance of the fungi with the low pH after

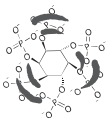
14 days incubation implicates an active microbial community as the source of the acidic conditions.

Despite differences in IP6 concentrations between the PL 14 and PL 1 treatments at 0 – 5 cm, there appears to be no difference in the mobility of the IP6 released from the poultry litter as indicated by the proportions of IP6 at the 3 depths in Table 6.2. This suggests that the IP6 is completely released from the poultry litter matrix, occurs in the same form in the soil and is equally amenable to transport after different incubation times. Whether the transport mechanism for IP6 is *via* the soil solution or as part of the particulate fraction was not determined due to the low concentrations of mobilised IP6 in this timeframe. This preliminary experiment indicates that the study of IP6 transport is best conducted after two weeks of incubation for maximum concentrations of soil IP6.

More replicates, treatments and timepoints would give an even better insight into the mechanisms at play. Both physical and biological processes appear to govern the recalcitrance, stability and mobility of the compound following application to the soil. The composition of poultry litter has a substantial impact on the availability of IP6 to extraction. There are implications for the availability of P from IP6 to plants and/or the soil matrix at different times post manure application and this may be relevant to the means of transport of IP6 from soil to watercourses, although more comprehensive study is required.

6.7 Conclusion

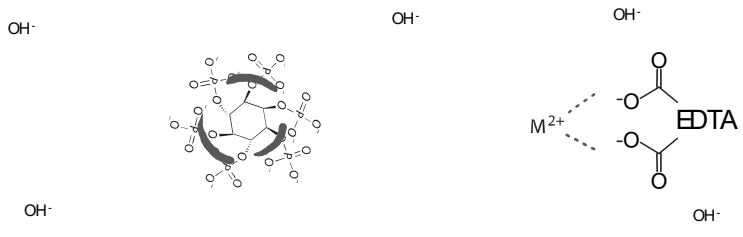
The experiments conducted here reveal an insight into the behaviour of IP6 from a natural source in soil. Using IC, a more rapid method than ^{31}P NMR spectroscopy, to quantify IP6 facilitated more time points and replicates and therefore more detail than in previous studies.



What was discovered was a dynamic behaviour of IP6 in soil in the weeks after application in poultry litter to soil.

1. Rather than the expected degradation from application of poultry litter to Week 11, there appears to be a large increase in the concentration of IP6 from Week 0 to Week 2, followed by a depletion after the peak at Week 2. The concentration of IP6 does not fall below initial concentration until Week 7.
2. IP6 in poultry litter is likely in a condensed recalcitrant form in protein complexes, and upon application to the soil, is released by biological activity. The concentration of IP6 therefore increases as the poultry litter is broken down, reaching a peak around Week 2. The concentration then decreases as the IP6 is degraded in the soil matrix.
3. Commercial IP6 in solution potentially exhibits a similar, but less pronounced behaviour when applied to soil. There is a slight increase in concentration from Week 0 to Week 2 followed by depletion over the following weeks. It is hypothesised that the compound is initially physically stabilised in the soil. Biological activity, such as the release of oxalate, may facilitate the release of IP6 from the soil matrix and make it available for digestion or degradation. Further studies are required to verify the phenomenon of increased concentration in the first weeks of incubation.
4. The transport experiment indicates that the concentration of IP6 from poultry litter is indeed elevated in soil after two weeks of incubation but is just as amenable to transport through the soil after one day incubation.
5. The appearance of fungi and the depressed pH in the upper level of the PL 14 and PL 1 soil column point to microbial activity breaking down the poultry litter and facilitating the release of IP6 from protein complexes.

These results may have implications for the contribution of IP6 from natural sources to soil P and the availability of the compound for P uptake or transport at different timescales post application of manure to soil. More detailed studies are required to determine the magnitude of these trends and the factors governing this dynamic behaviour of IP6 in soils.



Chapter 7: Synthesis and discussion

7.1 Introduction

The aim of this thesis was to develop and apply new analytical approaches that would provide insights, at the molecular level, into the role of organic P compounds in the P biogeochemical cycle; ultimately to inform management practices to minimise human impact on the cycle. The investigation of organic P compounds in environmental matrices has faced challenges due to: (i) their low abundance in nature relative to carbon and mineral compounds, (ii) their diversity of chemical properties (from phospholipids to IP6), (iii) the aggressive conditions required for extraction, and (iv) the technical challenges in instrumental analysis, such as the absence of a chromophore for UV detection, or the low resolution of spectra from ^{31}P NMR. HRMS combined with IC was chosen to overcome these challenges and characterise P at the molecular level in soil extracts. The key soil organic P compound IP6 was used as a model organic P compound for the development and application of the methodologies. Questions related to the behaviour of the compound in soil and manure were posed to test the application of the analysis method.

Overall, the results reported in this thesis provide a detailed, molecular level characterisation of the behaviour of IP6 in the environment and demonstrate the advantages of the newly combined IC and HRMS method for rapid characterisation of P compounds. The potential for its application to a range of questions, compounds, and environmental matrices is demonstrated. An overview of the main findings of this thesis is presented below. The results of preliminary investigations of the IC and HRMS behaviour of a range of other organic P compounds is discussed, leading to recommendations for future work.

7.2 Overview of work undertaken and major findings

7.2.1 Mass spectrometric analysis of IP6 and IP5 (Chapter 3)

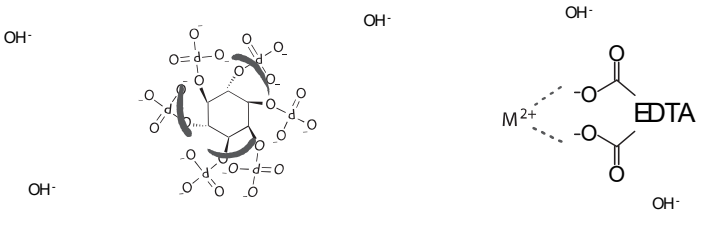
Using ESI-HRMS, combined with IC and MS/MS the behaviours of IPx reference compounds IP5 and IP6 were characterised. A pattern of charge acquisition, fragmentation and adduct formation in the ESI was identified. The fragmentation behaviour was verified *via* MS/MS experiments and a study of the effect of changing the source voltage on the composition of the mass spectrum. The influence of impurities of IP6 in the reference compound on the ion pattern in the full mass spectrum was discounted using purification *via* IC and the purity of the reference was thus determined.

7.2.2 Identification and quantification of IP6 using IC followed by ESI-HRMS v NMR (Chapter 4)

The separation of soil and manure extracts using IC and unequivocal identification of IP6 in the extract post-detection using ESI-HRMS was demonstrated. Ion suppression in the IC system produced an analyte solution which was low in salt and therefore unaffected by ion suppression in the ESI source. Standard addition was used to quantify IP6 in extract IC chromatograms and the quantification was compared to current NMR spectroscopic methods. Quantification of IP6 using both methods correlated well ($r = 0.9554$), validating the accuracy of the IC determinations. Compared to NMR spectroscopic analysis, this newly-developed method is much more sensitive with LOD of 0.7 mg kg^{-1} and LOQ of 2.1 mg kg^{-1}, more rapid with a quantification time of 6 h, and requires far less extract solution (200 µL v 5 mL).

7.2.3 Extraction efficiency of NaOH-EDTA method and contribution of soil characteristics (Chapter 5)

Using the standard addition in IC method of quantification of IP6 a range of three soil types (clay, alluvial, peat) under three different landcovers (arable, grassland, woodland) were spiked



with the compound prior to extraction and the extraction efficiency of the standard NaOH-EDTA method was determined. The main characteristics expected to contribute to the binding of IP6 to soil were measured for each soil type – metal concentration, organic content (LOI) and pH – and their correlation with EEIP6 were determined. It was found that there was a significant ($p < 0.001$), positive correlation between total metal concentration and EEIP6. A significant ($p < 0.001$) negative correlation was found for LOI and EEIP6. A less significant ($p < 0.05$) positive correlation was found for EEIP6 and Fe concentration. The extraction efficiency for total P was also measured and there were found to be no corresponding relationships between EETIP and the same characteristics as those found for IP6. It was concluded that the NaOH-EDTA extraction favours the extraction of IP6 versus total P in soils with high total metal concentration, particularly Fe and low organic content.

7.2.4 Fate of IP6 from poultry litter in soil incubation (Chapter 6)

The fate of IP6 applied to soil in a natural form – from poultry litter – and as a commercial IP6 solution was investigated in an incubation experiment with three replicates for each timepoint. Sampling was weekly for the first three weeks, then every second week until Week 11. It was found that, rather than degradation of IP6 post-application, there was a large increase in IP6 concentration in the soil amended with poultry litter. After this peak, the concentration in IP6 then declined over the following weeks. The average IP6 concentration remained above the initial concentration until Week 7. A similar, but much less pronounced trend was observed in the phytate solution treatment. It is suggested that IP6 in poultry litter is strongly bound with the proteinaceous compounds in the grain which pass through the poultry gut undigested, and is not amenable to extraction with NaOH-EDTA. Biological activity of soil microbes facilitates the release of the compound when incubated with soil. Once released, the compound is available for degradation and the concentration then declines. It is hypothesised that IP6 in

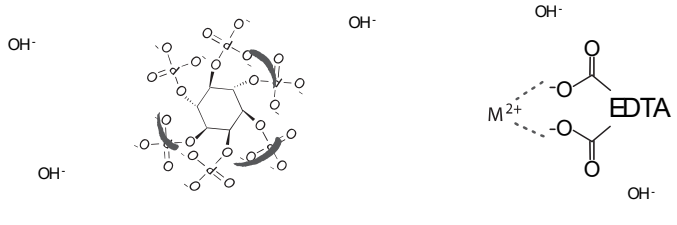
standard solution is initially physically immobilised in the soil and slowly released and degraded by biological activity over the first two weeks.

7.2.5 Transport of IP6 from a natural source through soil – preliminary leaching experiment (Chapter 6)

The design of the soil and poultry litter leaching experiment was informed by the results of the incubation experiment and soil columns were treated with poultry litter and pre-incubated for either 14 days or 1 day. The amended soil columns and a control were then leached until equilibrium flow and the concentrations of IP6 at three depths (0 – 5 cm, 5 – 10 cm, 10 – 20 cm) were determined. The results confirmed the observation of increased soil IP6 concentrations after 14 days incubation with poultry litter versus 1 day. Fungus at the top of the soil column on the 14-day treatment and pH measurements of the soil cores pointed to microbial-mediated activity in the breakdown of poultry litter and the release of IP6 for extraction and degradation.

7.2.6 Wider application of IC and HRMS to the identification and characterisation of known and unknown organic P compounds in environmental matrices

ESI-HRMS can resolve and identify multiple ions in a mixture, and so is a good prospect for in-depth analysis of organic P compounds leading to new insights. The challenges with implementing this technique for analysis of soil extracts are the complexity and the high ionic strength of extract solutions along with low P concentrations. Ion suppression in ESI-HRMS has been a major obstacle for the analysis of extracts and the introduction of ion chromatography for sample simplification proved essential. IC is particularly suited to the strongly ionic extract solutions as the ion suppressor in the IC flowpath removes excess salts from the analyte matrix enabling effective ionisation of phosphorylated compounds in the ESI.



The method can be applied in the same way to the characterisation of other organic P compounds from a range of matrices to aid our understanding of molecular level mechanisms in the P biogeochemical cycle. Some preliminary work has been done using other organic P reference compounds and modified soil extracts, along with the development of an automated method of identification of P compounds by the loss of phosphate in MS/MS experiments.

Reference compounds (Figure 7.1) were purchased and reference solutions prepared for IC and direct infusion ESI-HRMS. The reference compounds used were adenosine 3′ monophosphate (A3MP), adenosine 5′ diphosphate (A5DP), D-glucose-6-phosphate (DG6P), ethanolamine monophosphate (EAMP), guanosine 5′ monophosphate (G5MP), methylumbelliferyl phosphate (MUFP), phosphocreatine (PCRT), phosphoenolpyruvate (PEP). They were chosen, not only as known P compounds found in the environment (Turner *et al.,* 2003a), but also as they represent a range of organic P compounds with differing properties. For example, A3MP, A5DP and G5MP are nucleotides, A5DP has a diphosphate moiety and is substituted at a different position to A3MP, PCRT has a P-N bond, MUFP has two aromatic rings, one of which is bound directly to the phosphate, and EAMP is an amine.

Solutions of the reference compounds were prepared for HRMS at 10 ppm in DDW in furnaced amber glass vials. The ESI-HRMS negative ion mass spectra of each of the compounds was determined using the method in Section 2.10.2.2. The negative ion mass spectra of A3MP, A5DP, D6GP, EAMP, G5MP, MUFP, PCRT, and PEP are shown in Figure 7.2 A and B. Their mass spectra are a lot more straightforward than the IP6 negative ion mass spectrum, and in agreement with what should ordinarily be expected from ESI-MS, with minimal fragmentation and a dominant $[M–H]^-$ ion for most compounds. Four of the compounds (AD5P, G5MP, MUFP, PCRT) undergo fragmentation under ESI conditions with the loss of $[HPO_3]^-$ the most common form. A5DP readily loses $[HPO_3]^-$ to form an ion isobaric with that of the A3MP [M–

H]$^-$ ion. The A5DP [M–H]$^-$ ion is not present in the mass spectrum. A doubly charged ion of G5MP appears in the mass spectrum, however the [M–H]$^-$ ion is the dominant ion.

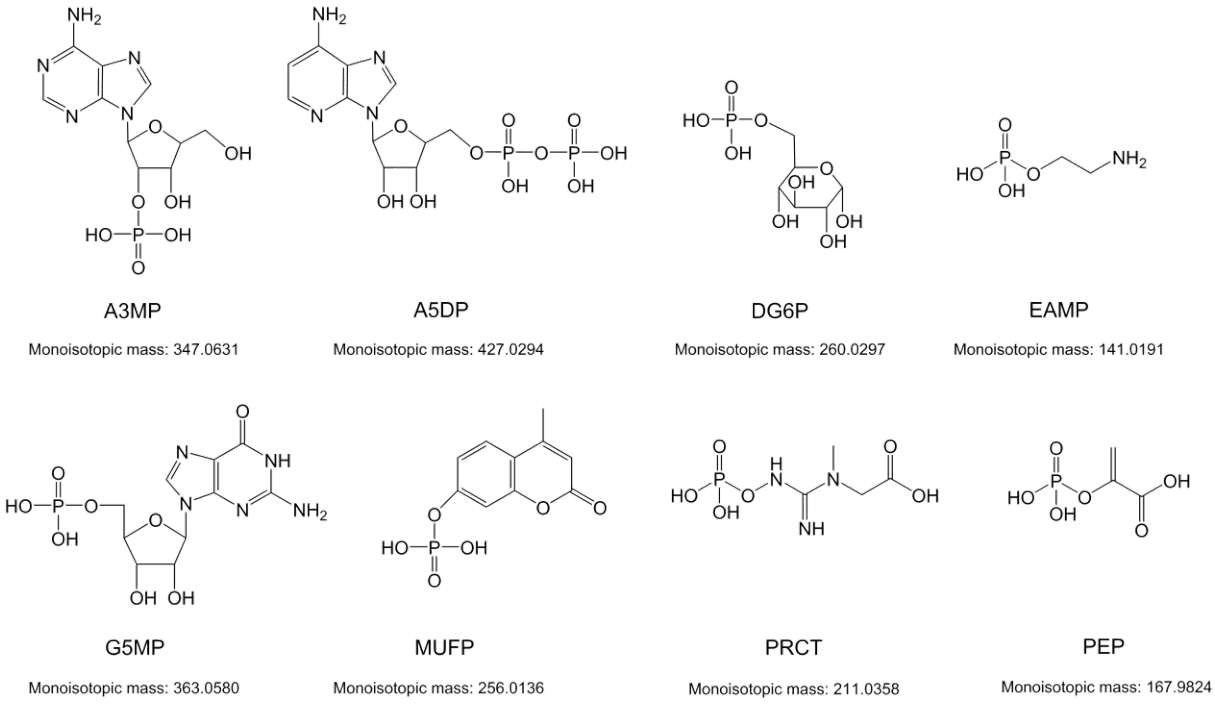
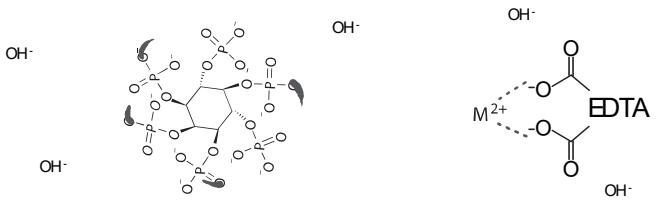


Figure 7.1. Structures of the eight reference compounds (A3MP, A5DP, DG6P, EAMP, G5MP, MUFP, PCRT, PEP) and their monoisotopic masses.

The ESI-HRMS negative ion mass spectra of reference compounds indicate that most organic P compounds behave in an expected way in the ESI with a dominant [M–H]$^-$ ion in the mass spectrum, minimal fragmentation or charge acquisition and no formation of salt adducts, as seen in the IP6 mass spectrum. Adjustment of conditions will be necessary to prevent fragmentation of A5DP as the loss of $[H_2PO_3]^-$ is complete for this compound. Otherwise MS/MS experiments may point to fragmentation patterns which can identify the different substitution patterns of the ribose sugar between A3MP and A5DP. MS/MS experiments were briefly attempted (data not shown), but conditions were not suitable for identification of



fragmentation patterns for all of the reference compounds and more experimentation is required.

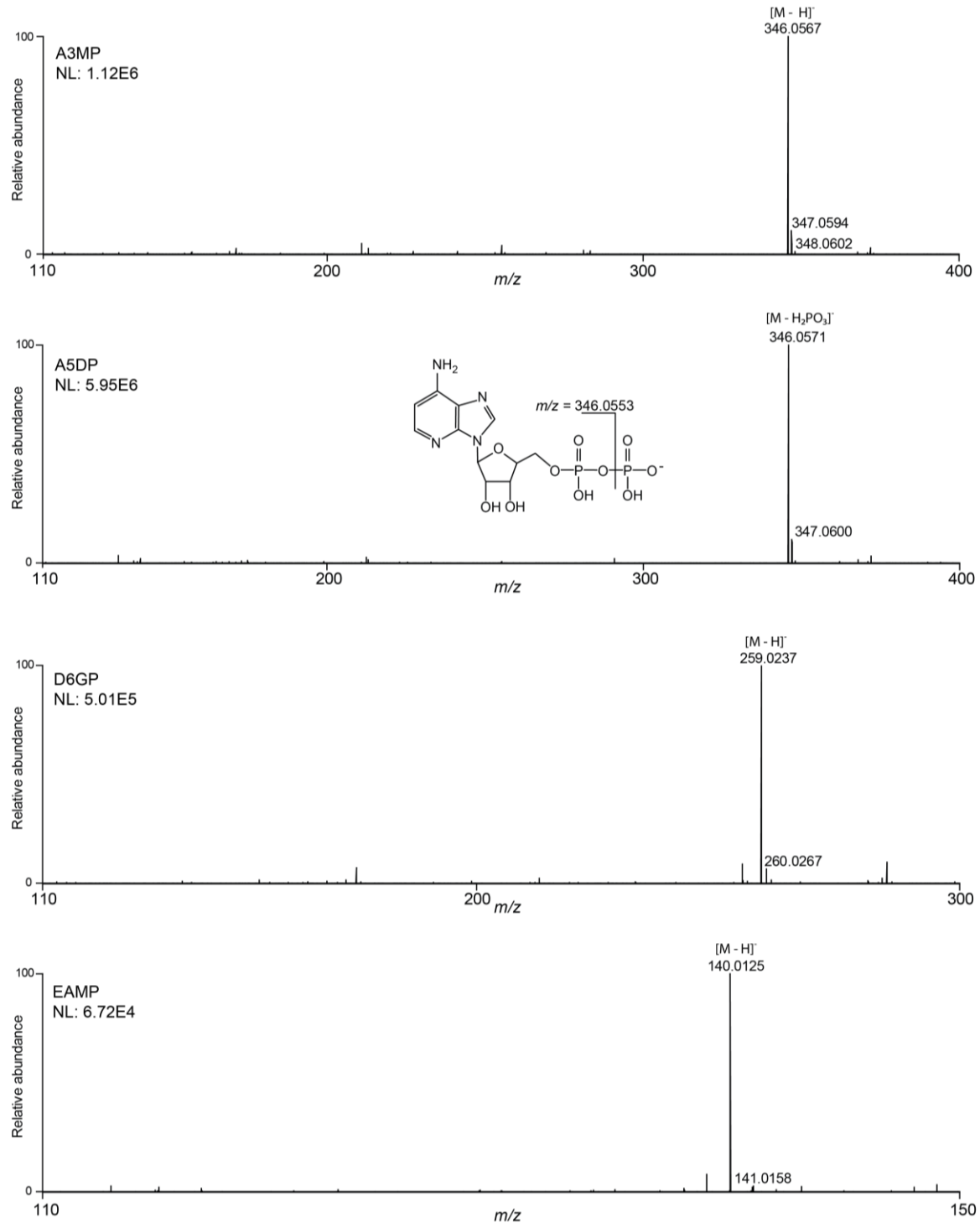


Figure 7.2 A. ESI-HRMS negative ion mass spectra of reference compounds A3MP, A5DP, D6GP and EAMP. The intensity of the most abundant ion is given by NL (normalised level).

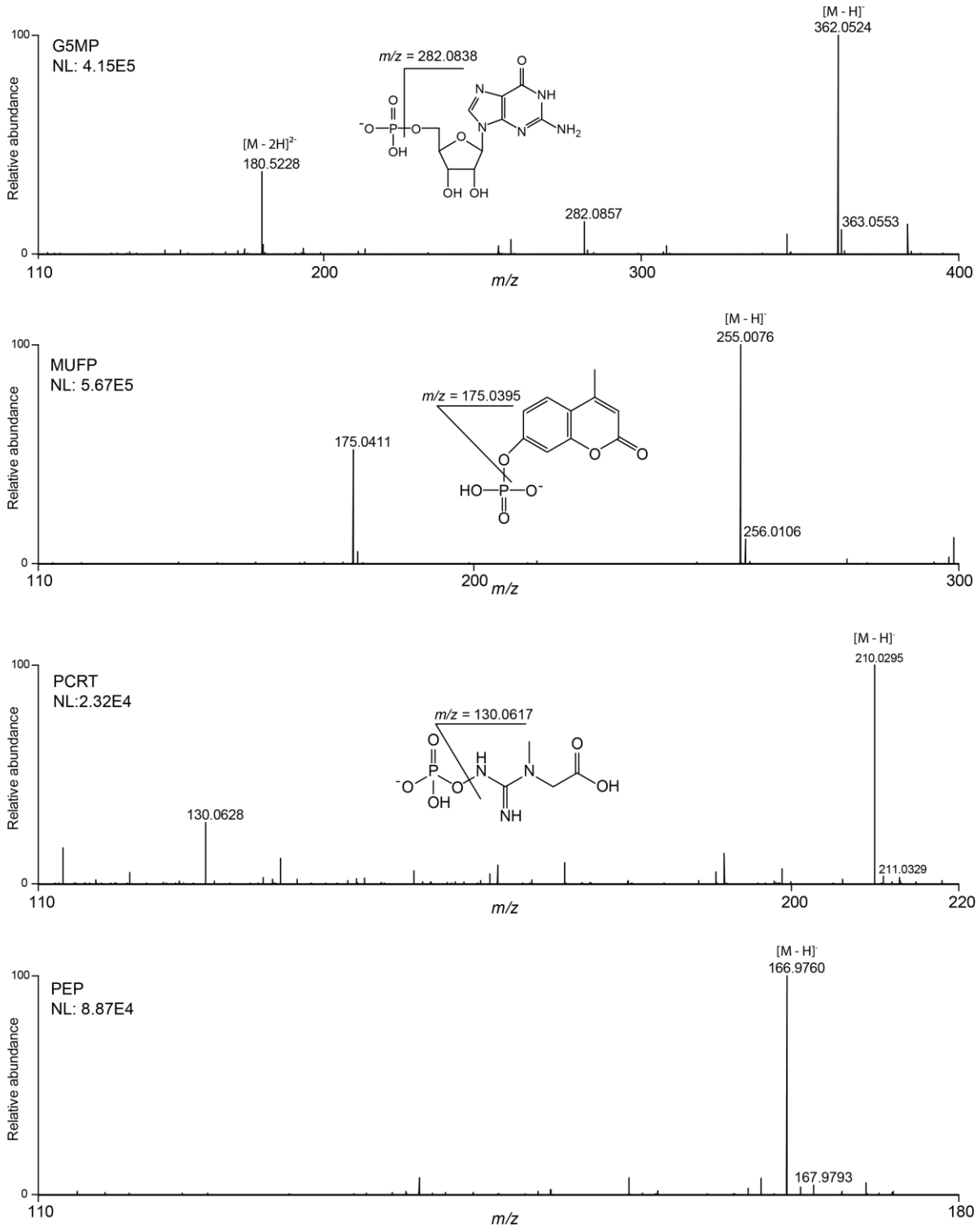
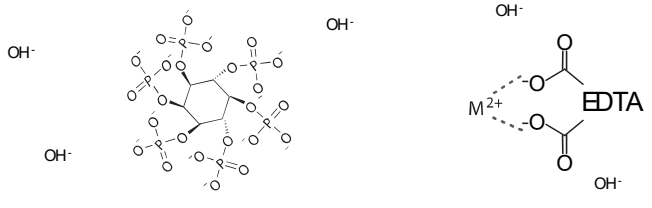


Figure 7.2 B. ESI-HRMS negative ion mass spectra of reference compounds G5MP, MUFP, PCRT and PEP. The intensity of the most abundant ion is given by NL (normalised level).



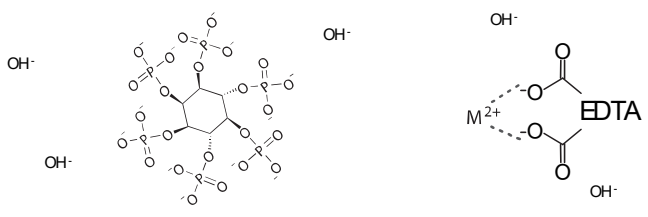
A soil was extracted using a modified two-step extraction procedure after Ahlgren *et al.* (2007). The soil was extracted using first a 0.067 M EDTA solution in a 1:3 w/v ratio for 30 min. After centrifugation (45 min at 3300 rpm), the EDTA extract was discarded and the soil resuspended in the same volume of 0.1 M NaOH for 16 h. After centrifugation and syringe filtration (0.42 μm), the extract was diluted twenty-fold to minimise NaOH concentration, spiked with the reference compounds, as well as IP6, at 20 ppm, and directly infused on the Orbitrap using the standard direct infusion method described in Section 2.10.2.2 and the MS/MS method described in 2.10.2.3. The MS/MS data was analysed using a custom-built program developed in R (see Appendix A2). The program searches the MS/MS data for fragment ions with *m/z* = 78.96 (PO_3^-), then generates a report detailing the corresponding precursor ion and its intensity in the full MS.

The full ESI-HRMS mass spectrum (between *m/z* 110 and 500) of the spiked soil extract is shown in Figure 7.3. The spectrum is dominated by, as yet, unidentified ions at *m/z* 164.0121, 216.9942, 351.0129, and 456.9772 and a number of other lower abundance ions. Given the complexity of soils (see Figure 1.11), there are surprisingly few ions in this spectrum. Reference ions identified by the R program are MUFP, and the ion corresponding to either the A3MP ($[M-H]^-$) or A5DP ($[M-H_2PO_3]^-$). These ions are at very low intensity (54 and 63 times less intense than their corresponding ions in the reference spectra) and are only visible on close inspection of the baseline of the full mass spectrum. The remaining reference compound ions are completely absent from the spectrum.

The small number of ions present in the ESI-HRMS negative ion mass spectrum of the modified soil extract indicates that the analysis is likely affected by ion suppression in the ESI source due to high concentrations of ionic species. Seven out of nine added reference compounds were absent from the mass spectrum, including IP6, and those were present

appeared at a fraction of the intensity expected for the concentration based on the reference mass spectra.

The analysis was conclusive evidence that direct infusion ESI-HRMS is an impractical approach to the analysis of soil extracts, despite the soft ionization technique and the ability of the Orbitrap mass spectrometer to resolve ions in mixtures. The MS/MS program used in the analysis of reference compounds can also be used for the detection of known and unknown P compounds in complex mixtures. It is a quick and automated way of detecting precursor ions to the PO_3^- ion in large MS/MS datasets. There were 84 P compounds identified in the soil extract, including the two reference standards. However, given the effect of ion suppression on the MS, this is certainly an inaccurate evaluation of the P content of the soil.



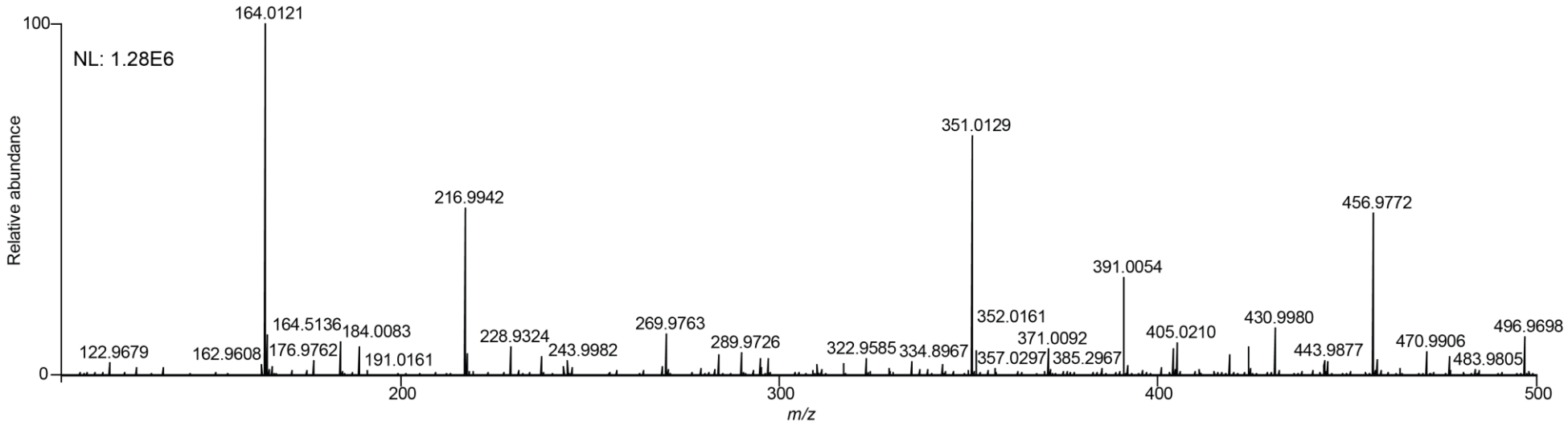


Figure 7.3. ESI-HRMS negative ion mass spectrum (*m/z* 110 to 500) of the modified soil extract containing 9 reference compounds. The absolute intensity of the most abundant ion is 73 and 124 times that of the identified compounds: A3MP/A5DP and MUFP, respectively. These ions are 63 and 54 times less intense than the reference compounds in the spectra in Figures 7.2 A and B.

7.2.6.1 Application of IC for the separation of organic P compounds

The reference compounds were dissolved in Milli Q water at concentrations of 120 µM for IC analysis. The IC conditions were as described in Section 2.10.1 – identical to those used to analyse IP6 and the soil extracts. The overlaid ion chromatograms of five reference compounds (EAMP, A3MP, PEP, G5MP and IP6) are shown in Figure 7.4. The compounds all elute at different times during the run and are well separated from each other. The chromatographic conditions were suitable for all reference compounds, except for EAMP which tails considerably.

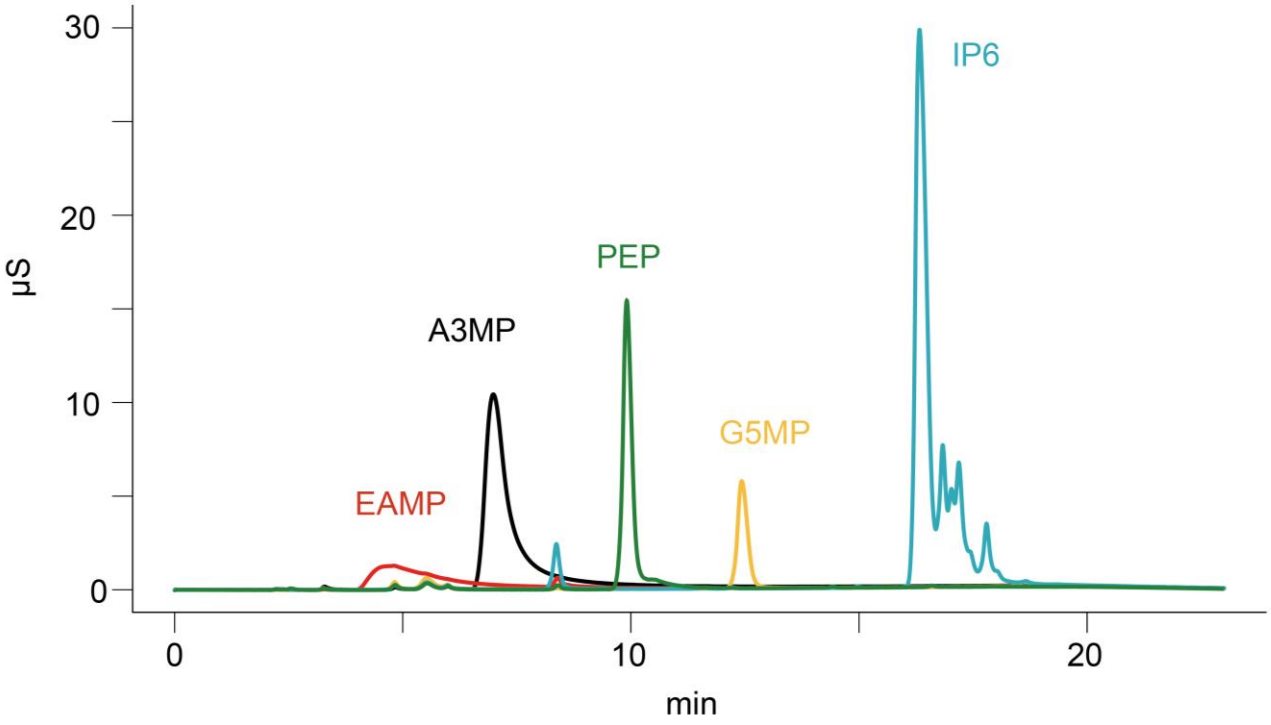
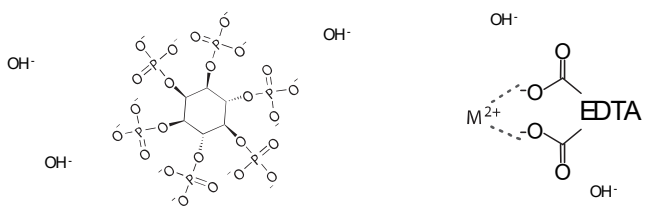


Figure 7.4. Overlaid ion chromatograms of five reference compounds: EAMP, A3MP, PEP, G5MP and IP6.

The IC results indicate that each of these compounds can be separated from each other when present in a mixture although some adjustment to the chromatographic conditions would be required for the analysis of EAMP. These data represent just one attempt at IC of these compounds and there are myriad options for optimisation of the process for separation of



known compounds. The reference compounds can be used to indicate the efficacy of different gradient conditions or timings on the separation of compounds and guide the process of improving the chromatography of soil and manure extracts.

This preliminary work with reference organic P compounds and a modified soil extract demonstrate that ESI-HRMS can be applied to the characterisation of many more organic P compounds. Ion suppression in direct infusion ESI-HRMS is incompatible with analysis of soil extracts. IC has been shown to be a viable option for the separation of organic P compounds and soil extract matrix compounds. A rapid method of identification of P compounds using programming in R has been demonstrated.

In Chapter 4 the problems identified with direct analysis of high ionic strength soil extracts in ESI-HRMS were overcome with the addition of IC, and IP6 was identified after isolation from a soil extract without need to modify the extraction procedure. The benefit of chromatography is the separation of matrix compounds, and therefore simplification of the mixture at the ESI source, and as discussed, the ion suppressor in the IC workflow enables good ionisation of compounds by eliminating suppression due to salts. This method renders otherwise undetectable soil compounds observable in the mass spectrum and identification of compounds is vastly more reliable, than for example in ^{31}P NMR spectroscopy.

7.3 Recommendations for future work

The method developed in this thesis, and the applications demonstrated, offer many avenues for further research and development. The IC and HRMS methods can be applied to answer questions related to IP6 in the environment and to the P biogeochemical cycle in general. The

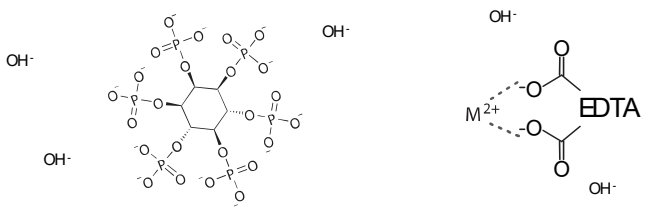
rapidity and precision of the method enables detailed investigation of mechanisms. Some applications identified during the course of this work are outlined here.

7.3.1 Connect IC with ion suppression to ESI-HRMS to study IPxs and organic P

Ideally, ion suppression IC would be connected directly to an ESI-HRMS. This would greatly improve the efficiency of analysis using this method. Real-time detection and fragmentation of compounds would be possible and multiple analyses could be performed in quick succession. This would accelerate the optimisation of the chromatography as discussed above. Online IC/HRMS would enable a much higher resolution in the time domain for analysis of closely eluting compounds, such as the inositol phosphates. Sensitivity could be improved for low concentration analytes using selected ion monitoring (SIM) with online IC/HRMS. As costs of HRMS instruments become more affordable and Orbitrap instruments become more common in analytical laboratories, these kinds of analyses will become more routine.

7.3.2 Modification of the EDTA concentration

One of the biggest obstacles to analysis of P compounds in soil extracts using IC, is the dominance of the EDTA peak in the chromatogram, limiting the amount of sample that can be loaded to the column. EDTA is used in vast excess in the extraction procedure. Experiments should be undertaken to determine if that concentration can be reduced – perhaps depending on the matrix properties – and the effect that has on total P and IP6 extracted. It was observed that soil types had different magnitudes of the EDTA peak in the chromatogram and that those with smaller EDTA peaks had a corresponding large, broad peak eluting prior to EDTA (Chapter 5, Figure 5.4). These soils were also the ones with higher calcium concentrations, with the largest broad peak found in the QHAR soil extract, which had the highest calcium concentration. We hypothesise that the broad peak represents EDTA bound to metals and that the typical EDTA peak at ~ 12 min is the remaining unbound EDTA. If this is the case, then



the EDTA is present far in excess of what is required and can be significantly reduced. Further study is required to prove or disprove this theory and determine the effects of the other metals on the form of EDTA in the chromatogram. Combining the chromatography with ICP-MS would enable determination and quantification of the metal ions at different stages of the chromatogram. A pre-column (Dionex Ionpac, CP2) that removes metal cations can be introduced prior to the anion exchange column to trap the metals and remove their impact on the chromatogram.

7.3.3 Optimisation of the NaOH-EDTA extraction method

The NaOH-EDTA extraction method has long been believed to be the most effective for extraction of organic P from soils (Cade-Menun & Preston, 1996). However, the method is not effective for all P compounds or for all matrices. As shown in this thesis, efficiencies can be as low as just 6 % for IP6 for a range of soils. It was also found that this method of extraction can result in over-estimation of IP6 relative to total P in soils with high metal content and low organic content, and under-estimation of IP6 in soils with low metals and high organic content. The relative proportions of the extraction chemicals, extraction time, sample preparation procedures can all be tested quickly and easily with quantification using IC. After filtration of extract and dilution for IC, preliminary results can be obtained within hours of an extraction, versus up to 72 h for ^{31}P NMR analysis.

7.3.4 Determination of the soil characteristics governing IP6 retention and extraction

Using a wider range of soils and conditions, a systematic review of the NaOH-EDTA extraction procedure and its efficiency for IP6 and total P in soils would inform not only the best method of extraction of the compound, but also give an insight into IP6-extraction solution interactions and IP6-soil interactions. Results could be compared with extractions using, for example HCl as per Waithasong *et al.* (2015), as well as other extraction solutions used in sequential

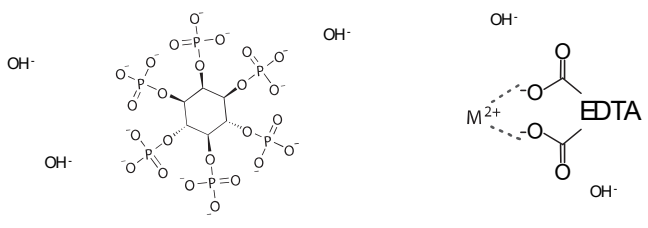
fractionation studies. In this way, a detailed depiction of the factors governing soil-IP6 interactions and the availability of the compound to biota could be built.

7.3.5 A high-resolution study of the first four weeks of IP6 in-soil incubation

The results of Chapter 6 indicate a dynamic behaviour of IP6 in soil in the first 4 weeks post application, and particularly in the first two weeks. A high-resolution study of the fate of IP6 – both from the poultry litter source and the phytate solution – in this critical period is required to understand the dynamics of its interaction with soil and the soil microbiota. Ideally, an initial study would involve either daily sampling, or every second day for the four weeks. This would confirm or refute the observed increase in IP6 concentration in the soil. This should be combined with observation of the soil characteristics e.g. soil pH, moisture content, TP, organic content in order to better understand the factors influencing the behaviour of the compound.

7.3.6 Investigation of the biological factors in the degradation of IP6 from manure in soil

To determine the contribution of biological and physical processes to this dynamic behaviour, the experiment should be repeated with an autoclaved soil. A separate incubation of poultry litter without soil, can help determine the contribution of microorganisms in the poultry litter itself, as opposed to the soil microbes. These studies could be combined with microbial ecology and molecular biology experiments. These techniques could be used to determine the quantity, diversity and activity of organisms contributing to the release and breakdown of IP6 in soil. The change in community and activity over time could then be traced over the course of the experiment and give insights into IP6 processing. More specific studies relating to the degradation of protein-bound IP6 are also recommended, perhaps using enzymatic hydrolysis of the protein-IP6 complexes to determine the effect on the release of IP6.



7.3.7 Determination of behaviour of IP6 from other sources

These experiments can then be replicated with differing sources of IP6 – for example from pig manure, cow manure or from sewage sludge used for fertilisation. This will provide more information about the dynamic behaviour of IP6 in soils and how that may be related to the diet or the animal from which the manure is sourced.

7.3.8 Further investigation of the transport of IP6 from natural sources

The preliminary lysimeter experiment indicated that IP6 from poultry litter is mobile under equilibrium flow, but that only small proportions of the total IP6 were mobilised. Using a longer simulated rainfall event, the full extent of IP6 transport in soil can be investigated. The leachate should be collected and filtered to determine how much IP6 is transported in the dissolved fraction, and how much in the particulate fraction. The low LOD and LOQ of IC indicate that the detection of the compound in leachate and small quantities of leached sediment is viable using this method.

7.3.9 Wider application to agriculture and aquatic systems

The sensitivity of IC for detection and quantification of IP6 demonstrates the potential for use of the method in systems with low concentrations of organic P, such as freshwater and marine systems. A preconcentration step such as solid phase extraction may be necessary for characterisation of P compounds in the water column, but sediment and particulate fractions can be extracted in the same way as that for soils. Using this method P cycling in aquatic, as well as terrestrial ecosystems, can be studied in molecular detail and contribute to our understanding of the role of organic P compounds, from anthropogenic as well as natural sources, in the global P biogeochemical cycle, and therefore inform the management of human influence on agriculture and water quality.

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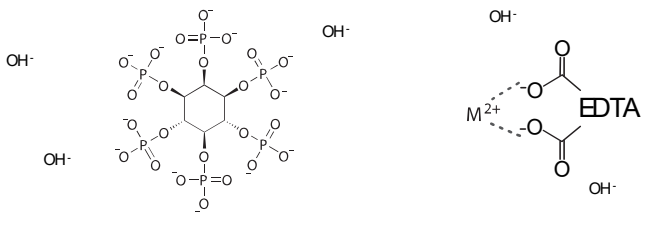

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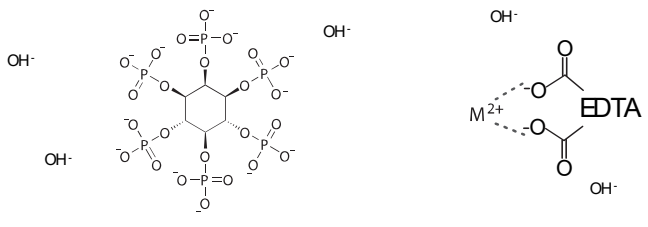

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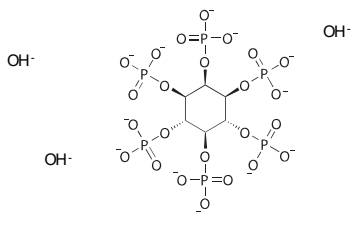

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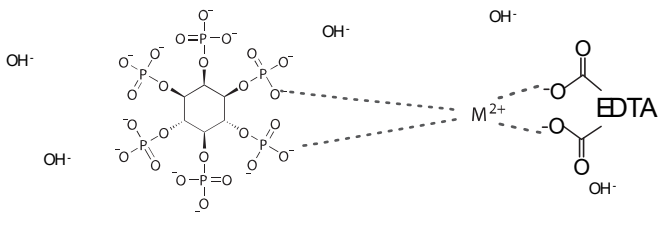

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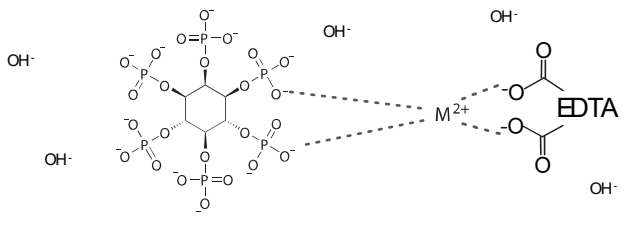

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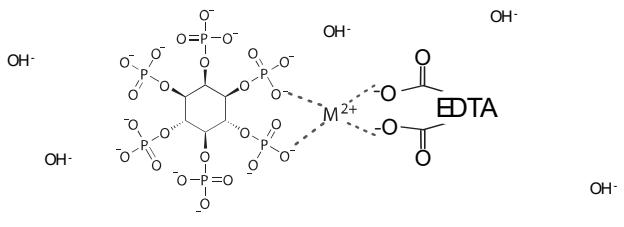

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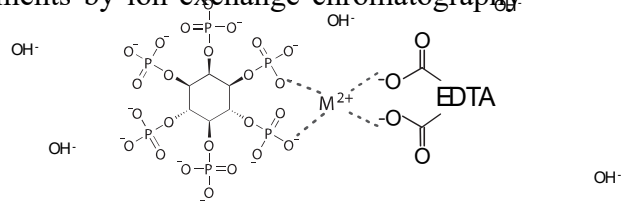

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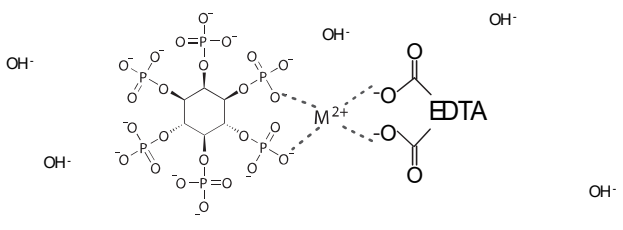

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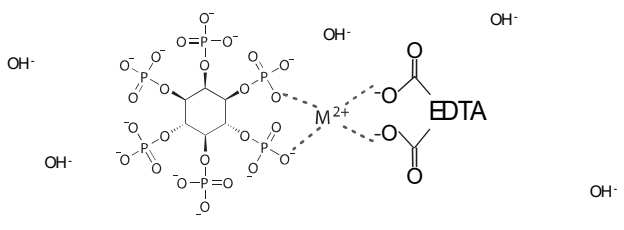

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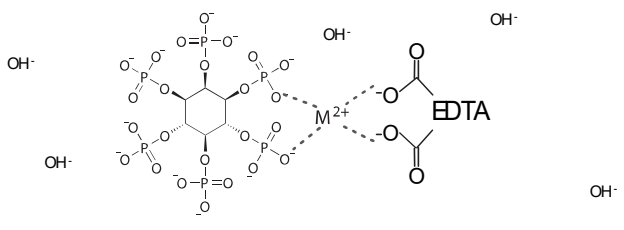

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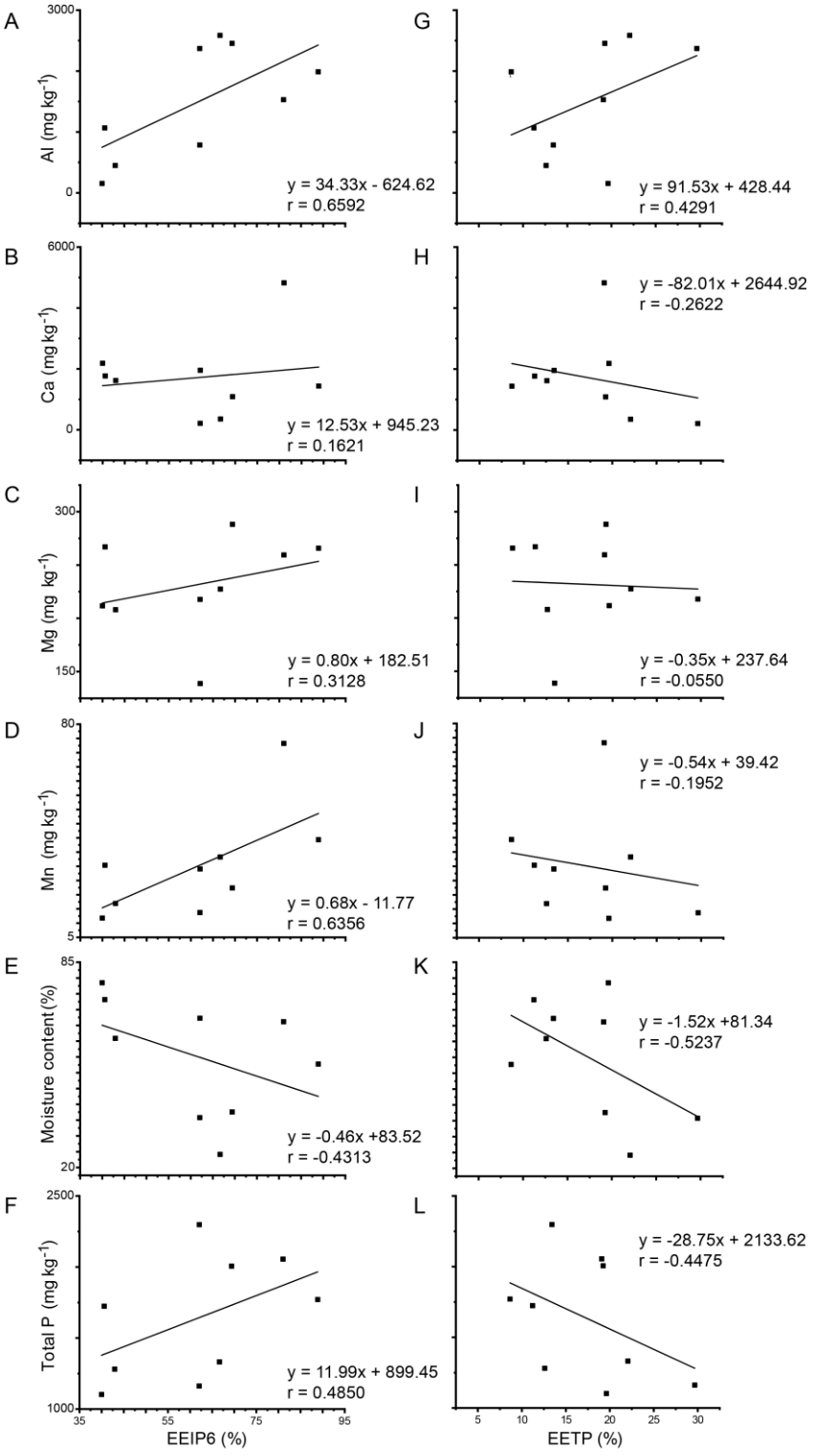
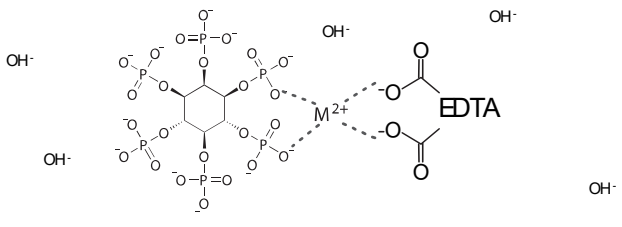


Figure A1.1. Pearson's product moment correlations between EEIP6 (A – F) and Al, Ca, Mg, Mn, moisture content and Total P. Correlations between EETP (G – L) and Al, Ca, Mg, Mn, moisture content and Total P. These correlations were not statistically significant at the $p \leq 0.05$ level.



A2 R Program

Aim:

Identification of PO_3^- fragment in MS/MS data, and plot MS of PO_3^- containing ions only.

Code

```
## 14 January 2016
# code imports mzXML ms file, isolates peak lists for each scan, then finds the PO3 fragment
# reports presence of 78.96 for each scan

# load package
library(mzR)

MSfile <- openMSfile(file.choose())
Scanswpeaks <- peaks(MSfile)

# this is print(Peaklist [scan number], [[table number]], [,column number]) to look at individual scans
## print(Scanswpeaks[3][[1]][,1])

# set x as vector with length same as peaks Scanswpeaks
x <- length(Scanswpeaks)

# create empty column PO3Yes
PO3Yes <- c()

# makes a vector that is the first column in each scan (table 1)
# rounds each value in that vector to 2 decimal places and creates a new vector
# finds 78.96 in rounded vector. puts that in to a new vector so it won't get overwritten.
i=1
for(i in 1:x){
  ioncol <- Scanswpeaks[i][[1]][,1]
  ionsround <- round(ioncol, digits=2)
  fragmentPO3 <- 78.96%in%ionsround
  PO3Yes[i] <- fragmentPO3
}

print(PO3Yes)

PO3column <- as.data.frame(PO3Yes)


##here's how to get the metadata
headerinfo <- header(MSfile)

headertable <- as.data.frame(headerinfo)

## put precursor mz, charge and intensity next to list of presence/absence of po3
scantable <- cbind(PO3column, headertable[,15:17])

##remove the precursor charge column
```

```
scantable <- scantable[,-3]

## only scans that are TRUE
attributes(scantable)
scantrue <- scantable[!(scantable$PO3Yes==FALSE),]

MSplot <- plot(scantrue[,2], scantrue[,3], type="h", xlim=c(125,800), ylim=c(0,1300000), col="268",
main="Precursor Ion Scan PO3-", xlab="m/z", ylab="Intensity")


write.csv(scantrue, file.choose())

MS1plot <- plot(Scanswpeaks[1][[1]][,1], Scanswpeaks[1][[1]][,2], type="h", xlim=c(125,800),
ylim=c(0,1300000), main="MS1 Scan", xlab="m/z", ylab="Intensity")
print(Scanswpeaks[1][[1]][1:20,])

scan2 <- as.data.frame(Scanswpeaks[2])
write.csv(scan2, file.choose())

print(Scanswpeaks[240])

colors(plot.it=T)

MS1data <- as.data.frame(Scanswpeaks[1])
write.csv(MS1data, file.choose())
```